Evaluation of anti-bovine, anti-equine and recombinant protein A/G horseradish peroxidase conjugates for cross reactivity to wildlife serum antibodies using ELISA

by

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Declaration of originality

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Table of contents

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List of abbreviations

Ab	Antibody/antibodies
Ag	Antigen
AGID	Agar gel immunodiffusion test
CB	Carbonate/bicarbonate coating buffer
CFT	Complement fixation test
CT	Chaotrope
EIA	Enzyme immune assay
ELISA	Enzyme linked immunosorbent assay
bELISA	blocking ELISA
cELISA	competitive ELISA
dELISA	direct ELISA
iELISA	indirect ELISA
isELISA	indirect sandwich ELISA
Fab	Antigen binding fraction of the antibody
Fc	Crystallisable fraction of the antibody
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IgG	Immunoglobulin G
M	Molar (concentration with unit mol/L)

MAb	Monoclonal antibodies
OD	Optical density
OPD	o-Phenylenediamine dihydrochloride
PAb	Polyclonal antibodies
PBS	Phosphate buffered saline (0.15 M PBS, pH 7.22)
PBST	PBS with 0.05% Tween 20
RAI	Relative avidity index
RPM	Revolutions per minute
SP	Sample to positive
UI	Usefulness index

List of tables

List of figures

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Abstract

Primary binding assays like the indirect immunofluorescence assay or the enzyme-linked immunosorbent assay (ELISA) are serological methods that have been used to effectively screen for many diseases in humans and animals. These assays depend on reagents that can recognise antibodies (immunoglobulins) of the target species. The indirect ELISA for example requires an enzyme conjugated anti-immunoglobulin, which is specific to the target species, also referred to as an anti-species conjugate. Conjugated anti-species immunoglobulins are commercially available for all common domestic species and even for the predominant wildlife species in the northern hemisphere. The African wildlife species are, however, extremely diverse and anti-species immunoglobulins are not readily available, which renders primary binding assays that depend on these conjugated molecule largely unavailable for these species.

The purpose of this study was to evaluate the cross-reactivity of commercially available polyvalent anti-bovine IgG: HRP and anti-horse IgG: HRP and binding of recombinant protein A/G: HRP with antibodies in the serum of various herbivore African wildlife species. Serum from 27 herbivore and hoof-stock wildlife species were obtained and a direct ELISA was performed on 10 animals from each species for each of the three selected conjugated molecule. Binding reactions between wildlife serum immunoglobulins and rabbit anti-bovine: HRP, as well as recombinant protein A/G: HRP were expressed relative to the response to bovine serum immunoglobulin and cross-reaction with the anti-horse IgG: HRP was expressed relative to equine serum immunoglobulin. A relative affinity index as well as a usefulness index was calculated for each of the conjugated molecules for each of the wildlife species.

Thirteen wildlife species performed better than or equal to the bovine control with the recombinant protein A/G: HRP while the rest of the species performed significantly lower than the bovine control. In contrast, the conjugated rabbit anti-bovine IgG: HRP -was found not to be useful for any of the wildlife species tested in this study. The goat anti-horse IgG: HRP was found to bind equivalent to the equine control in four species, however the enzyme-conjugated recombinant protein A/G: HRP performed better in all four these species. The calculation of a usefulness index using the colour change (optical density) in relation to a known homologues control serum and the relative avidity index (a ratio of the measured effect of a chaotropic agent) could become a useful tool for evaluating and comparing the cross-reactivity between conjugated anti-species immunoglobulins or binding of other conjugated molecule with the antibodies in the serum of heterologous species.

Chapter 1 Introduction

1.1 Problem statement

Disease causing organisms (pathogens) or toxins that enter the body usually stimulates the immune system. Any such molecule that is able to stimulate an immune response and subsequently result in the production of antibodies is known as an antigen (Ag). Research over more than a century revealed that antibodies proved to be extremely valuable, not only to combat disease in the body, but also for diagnostic purposes. Serology, which is the identification of antibodies against a given Ag in serum (Washington, 1996, Ryan and Ray, 2004), was subsequently developed to indirectly confirm that a pathogen or toxin is or was present in the body. This can be determined either qualitative by the mere presence of antibodies and/or quantitatively by measuring the amount of antibodies in the serum (titre).

Serology initially included techniques such as the complement fixation test (CFT) and the Agar-gel immuno-diffusion (AGID) test, which was used in African wildlife serology during the 1980's (Blackburn and Swanepoel, 1988), but these methods are time consuming with lower throughput and more prone to subjective interpretation. A serological technique developed during the 1970's, namely the enzyme-linked immunosorbent assay (ELISA) has been used for the past 25-30 years to effectively screen for many diseases in humans and animals. It was later modified to use known antibodies to demonstrate the antigen in serum directly, as an antigen-capture ELISA. The ELISA is more sensitive, specific and much faster than older techniques allowing for high-volume throughput, and safe to use, without requiring radio-active substances used in comparable

older techniques (Lequin, 2005). The ELISA however requires a “conjugate”, which in most instances is an antibody binding with antibodies of the target species also known as an *anti-species immunoglobulin*. It is attached (conjugated) to an enzyme, which can be used to indicate or visualise successful binding.

The ability to recognise antibodies of a target species is extremely valuable in directly assessing an immune response using an ELISA and equally so the lack of an ability to recognise species-specific Ab, using an anti-species immunoglobulin is very limiting, leading to cumbersome measures such as the use of mice as a biological confirmation of successful immunisation (Turnbull et al., 2004) or the use of peptide ELISA assays to confirm a specific pathogen (Abdelgawad et al., 2015) or the use of indirect or competitive ELISA techniques to indirectly demonstrate an immune response (Afshar et al., 1987, Anderson, 1984).

These species-specific antibodies are produced in other species, for example, if rabbits are injected with cattle antibodies (IgG) they will develop anti-cattle IgG antibodies that can be used as a conjugate for ELISA assays on cattle serum (Abcam, 2010). Conjugates are commercially available for all common domestic species (Bio-Rad, 2017) and even for the predominant wildlife species in Europe namely deer (Rossi et al., 2014).

The African wildlife species are however extremely diverse and production of species-specific antibodies are not feasible. There is therefore currently no species-specific conjugates available for any of our African wildlife species, which currently renders the very useful ELISA assay largely unavailable for African wildlife.

Non-species-specific proteins of bacterial origin such as protein A, G and L can alternatively be used. These proteins represent very clever mechanism of the bacterium to

evade the host immune response by attaching to epitopes at the tail end of the antibody (Fc-fraction). They are, however, not species-specific when binding with antibodies, with a broad published species binding capacity (ThermoFisherScientific, 2017).

This study will test the cross-reactivity and affinity (avidity) of commercially available enzyme conjugated bovine and equine specific immunoglobulins (Bio-Rad, 2017, Sigma-Aldrich, 2017, Alpha Diagnostic, 2017) as well as a non-species-specific recombinants protein A/G: HRP (ThermoScientific, 2016) against a variety of African hoofed and herbivore wildlife species.

The purpose is to identify candidate conjugates that can possibly be used in future for ELISA development for specific wildlife species to test for numerous pathogens and antigens of in order to advance diagnostic and research capabilities feasibly and significantly. Effective ELISA for wildlife could be instrumental in clarifying aspects of the epidemiology of complex infectious diseases, which are still unclear despite major advances in infectious disease control over the past 120 years (Thrusfield, 2013). This could include the role of subclinical or asymptomatic wildlife carriers as a reservoir for vector borne livestock diseases such as blue tongue (Barnard, 1997, Gerdes, 2004, Steyn et al., 2015), which is of global concern (Lorca-Oró et al., 2014, Rossi et al., 2014, Meroc et al., 2009).

1.2 Aim and objectives

- To evaluate commercially available cross-reactive conjugated anti-species immunoglobulins and binding with non-species-specific protein A/G: HRP that

can potentially be used in ELISA assays for various African herbivore wildlife species.

- To quantify the usefulness of promising candidate conjugates.

1.3 Hypothesis

Null hypothesis:

Commercially available anti-species immunoglobulin enzyme conjugates will not cross-react differently with Southern African hoof-stock, herbivore wildlife sera immunoglobulins in ELISA assays, compared to the control species.

Alternate hypothesis:

Commercially available anti-species immunoglobulin enzyme conjugates will cross-react differently with Southern African hoof-stock, herbivore wildlife sera immunoglobulins in ELISA assays, compared to the control species.

1.4 References

ABCAM 2010. Understanding secondary antibodies. *In:* ABCAM (ed.).

ABDELGAWAD, A., HERMES, R., DAMIANI, A., LAMGLAIT, B., CZIRJÁK, G. Á., EAST, M., ASCHENBORN, O., WENKER, C., KASEM, S., OSTERRIEDER, N. & GREENWOOD, A. D. 2015. Comprehensive Serology Based on a Peptide ELISA to Assess the Prevalence of Closely Related Equine Herpesviruses in Zoo and Wild Animals. *PLoS ONE,* 10**,** e0138370.

AFSHAR, A., THOMAS, F. C., WRIGHT, P. F., SHAPIRO, J. L., SHETTIGARA, P. T. & ANDERSON, J. 1987. Comparison of competitive and indirect enzyme-linked immunosorbent assays for detection of bluetongue virus antibodies in serum and whole blood. *J Clin Microbiol,* 25**,** 1705-10.

ALPHA DIAGNOSTIC, I. 2017. Rabbit Anti-Deer IgG (H+L chain) (Cervid family: Deer, Elk, Moose)-Biotin Conjugate. Online: Alpha Diagnostic International Ltd.

ANDERSON, J. 1984. Use of monoclonal antibody in a blocking ELISA to detect group specific antibodies to bluetongue virus. *J Immunol Methods,* 74**,** 139-49.

BARNARD, B. J. 1997. Antibodies against some viruses of domestic animals in southern African wild animals. *Onderstepoort J Vet Res,* 64**,** 95-110.

BIO-RAD. 2017. *Helpful ELISA Hints* [Online]. Online: Bio-Rad. Available: https://www.bio-rad-antibodies.com/helpful-elisa-hints.html [Accessed 14 July 2017 2017].

BLACKBURN, N. & SWANEPOEL, R. 1988. Observations on antibody levels associated with active and passive immunity to African horse sickness. *Tropical animal health and production,* 20**,** 203-210.

GERDES, G. 2004. A South African overview of the virus, vectors, surveillance and unique features of bluetongue. *Vet Ital,* 40**,** 39-42.

LEQUIN, R. M. 2005. Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). *Clin Chem,* 51**,** 2415-8.

LORCA-ORÓ, C., LÓPEZ-OLVERA, J. R., RUIZ-FONS, F., ACEVEDO, P., GARCÍA-BOCANEGRA, I., OLEAGA, Á., GORTÁZAR, C. & PUJOLS, J. 2014. Long-Term Dynamics of Bluetongue Virus in Wild Ruminants: Relationship with Outbreaks in Livestock in Spain, 2006-2011. *PLOS ONE,* 9**,** e100027.

MEROC, E., HERR, C., VERHEYDEN, B., HOOYBERGHS, J., HOUDART, P., RAEMAEKERS, M., VANDENBUSSCHE, F., DE CLERCQ, K. & MINTIENS, K. 2009. Bluetongue in Belgium: episode II. *Transboundary and emerging diseases,* 56**,** 39-48.

ROSSI, S., PIOZ, M., BEARD, E., DURAND, B., GIBERT, P., GAUTHIER, D., KLEIN, F., MAILLARD, D., SAINT-ANDRIEUX, C. & SAUBUSSE, T. 2014. Bluetongue dynamics in French wildlife: exploring the driving forces. *Transboundary and emerging diseases,* 61.

RYAN, K. & RAY, C. 2004. Sherris medical microbiology: an introduction to infectious diseases. 4th. McGraw Hall USA.

SIGMA-ALDRICH. 2017. *Secondary Antibodies, Conjugates and Kits* [Online]. http://www.sigmaaldrich.com: Sigma-Aldrich. Available: http://www.sigmaaldrich.com [Accessed 16 February 2017 2017].

STEYN, J., VENTER, G., COETZEE, P. & VENTER, E. 2015. The epidemiology of bluetongue virus in Mnisi, South Africa. *American Journal of Epidemiology and Infectious Disease,* 3**,** 95-102.

THERMOSCIENTIFIC 2016. Binding characteristics of antibody-binding proteins: Protein A, Protein G, Protein A/G and Protein L. *In:* THERMOSCIENTIFIC (ed.). Online: ThermoScientific.

THRUSFIELD, M. 2013. *Veterinary epidemiology,* Great Brittain, Elsevier.

TURNBULL, P. C. B., TINDALL, B. W., COETZEE, J. D., CONRADIE, C. M., BULL, R. L., LINDEQUE, P. M. & HUEBSCHLE, O. J. B. 2004. Vaccine-induced protection against anthrax in cheetah (Acinonyx jubatus) and black rhinoceros (Diceros bicornis). *Vaccine,* 22**,** 3340-3347.

WASHINGTON, J. A. 1996. Principles of Diagnosis: Serodiagnosis in: Baron's Medical Microbiology. *In:* BARON, S. (ed.) *Medical Microbiology.* Fourth ed. Galveston, Texas: Univ of Texas Medical Branch.

Chapter 2 Literature review

2.1 History of serology and the enzyme-linked immunosorbent assay (ELISA)

In the late 1700s Edward Jenner numerously tested and documented the observed tendency of most people not contracting small pox or cow pox after earlier exposure to either, by re-exposing them. He also carefully documented his first experiment when he inoculating a healthy, previously unexposed child with cow pox and challenged him six weeks later with small pox inoculum, to which he proved protected (Jenner, 1800). This well-known case study marked the beginning of modern day vaccination and immunology (Allsopp et al., 2004). Since the 1880's with the acceptance of the microbial theory, control of infectious diseases became dependent on the isolation of disease-causing agents in a laboratory (Thrusfield, 2013). A vital component of survival is, however, the ability of living organisms to resist disease by mounting an intricately designed immune response and in 1890 antibody activity was first demonstrated. This was soon followed by the so-called *serum therapies* that used serum from a previously exposed individual for antibody transfer or passive immunisation (antiserum) (Abutarbush, 2008) that earned Behring the first Nobel prize in Physiology and Medicine in 1901 (Nobel, 2014).

All of this was instrumental in the effective control and/or the eradication of many infectious diseases in developed countries by the mid twentieth century. From nine of the top ten killers in the world being infectious diseases in 1860, apart from pneumonia-influenza-bronchitis, infectious diseases had virtually disappeared from the list of ten leading causes of death among humans by 1970 (Thrusfield, 2013). Due to their ability to bind with high specificity and in many cases with a high degree of affinity, antibodies (Ab)

have since been explored and developed through scientific discoveries and research to contribute significantly to the health and welfare of man and beast today. (Lipman et al., 2005).

Ab are large protein molecules known as immunoglobulins (Ig) that consist of two identical heavy and two identical light protein chains bonded together. The Y-shaped configuration consists of two highly variable but identical antigen binding sites on the Fab "arms", each with the capability to bind with a specific antigen (divalent), which is supported by the crystallisable fraction or Fc "base" portion, which is structurally considered as constant and has sites for binding with effector cells of the immune system, such as lymphocytes, to enable their functions (Hajela, 1991, Lipman et al., 2005). The Fc portion consists of two identical heavy protein chains, which are characteristic for each class of immunoglobulin. The different classes such as IgM (containing a μ-protein chain), IgG (γ-protein chain), IgA (α-protein chain) and IgE (ε-protein chain) are dominant at characteristic stages of the immune-response and their numbers are regulated accordingly (Tizard, 2013) p167.

The heavy chain consists of several constant domains, with different functions, including the FcR-binding region. Due to minor mutations in the conserved regions of the heavy chain, different subclasses of the different Ig heavy chains are recognised, but all subclasses are reported to be present in all individuals of each species (isotypes), for example IgG has three subclasses documented in cattle and sheep whereas horses have seven and pigs have six IgG subclasses. Structural inherited variation of the heavy chain of the Ig molecules between individuals are, however, described; for example, some individuals in a population may have IgG1(A1) and others IgG1(A2), which are referred to

as allotypes. Other structural variations on the antibody are limited to the variable regions of the light and heavy chains at the antigen binding sites and they include a large number of different idiotypes for every individual animal (Tizard, 2013, Lipman et al., 2005).

Size matters and, depending on the size of an organism or molecule that stimulates the immune response, also known as the *antigen* (Ag), a few to many diverse clones of antibodies (Ab) can be produced at once (polyclonal Ab), each capable of binding with a different place or epitope on the multivalent Ag in varying degrees of affinity. The strength with which binding of an Ab with an Ag is a product of the affinity (Ka) at a binding site and the number of binding sites (valence) with IgG having two and IgM having 10 binding sites (Kindt et al., 2007). The combined binding capacity of all these clones at varying affinities is known as the *avidity* of the polyclonal antiserum (Hudson and Hay, 1989). When an Ab binds exclusively with a unique epitope in a specific molecule or species, it is considered to have a high specificity. If, however, it binds with similar epitopes in different molecules or species, it is considered to be cross-reactive (Bio-Rad, 2017).

But Ab, themselves being large protein molecule of about 150kDa, are also capable of stimulating an immune response and when injected into an animal of a different species, Ab against the foreign Ig will be produced in the second species. Mice, rabbits, domestic small stock and chickens are often used as the second species. The further the two species are phylogenetically separated, the larger the number of foreign epitopes on the injected Ig that could stimulate an immune response in the second species. Closer related species in contrast have more similar or shared epitopes (Lipman et al., 2005). Through this process Ab are raised against the different fractions of the injected Ig, including the Fc portion (Kindt et al., 2007). These secondary antibodies are also called *antiglobulins* (Tizard, 2013).

As these antiglobulins are in essence species-specific, they can be used to identify the species of an antibody and are therefore very valuable reagents.

Although the majority of secondary antibodies binds with the constant region of the Fc portion of the primary Ab, also known as the isotype, which is common in all animals of that species, some recognise different allotypes, which may vary between animals of the same species and some may be directed at the variable domain of the antigen binding (Fab) site (idiotypes). When the secondary Ab is raised specifically against the heavy chain and the Fc portion they will only bind with that specific Ig class, but Ab raised against the whole IgG molecule or to the Fab usually reacts with all Ig classes. Some epitopes are, however, also found on Ig of other species, these secondary Ab may also cross-react with IgG from those species (Kindt et al., 2007).

The ELISA is an innovative and versatile immunodiagnostic laboratory technique that makes use of enzyme-labelled antiglobulins. Lequin (2005) traced the origin of the enzyme immune assays (EIA) and ELISA techniques from the late 1960s, when radioactive labelling of antigens were taken to a new level with radioactive labelling of antibodies in 1968. Due to the health and safety risks associated with using radioactive compounds in the laboratory, two independent research groups managed to implement the inconceivable idea of using a much safer option, namely the labelling of antibodies by chemically linking it to an enzyme instead, towards the end of that decade. During the 1970s work on the enzyme label techniques flourished and in the early 1980s the number of publications on the EIA/ELISA overtook those of radio immune assays (RIA), which dropped significantly after 1990. (Lequin, 2005).

Different configurations or variations contribute to this versatile serological test, where the Ag is coated or captured directly or indirectly on a solid surface, where it is then detected by an Ag-specific primary Ab, which is then signalled by an Ab-specific secondary Ab. The simplest form is the direct ELISA, which is seldom used due to low sensitivity and it should therefore only be used when the target Ag is relatively abundant. In such a configuration the Ag adheres to the plate (solid phase) and a conjugated primary Ab is used to bind with the Ag to signal the binding. (ThermoFisherScientific, 2010, Tizard, 2013).

With the indirect ELISA (iELISA) the Ag is detected by a primary Ab of interest present in the test serum and signalling is by a secondary Ab reagent, which is specific to the primary Ab. But even more layers can be added and the indirect sandwich ELISA (isELISA) with an additional Ag-capture Ab reagent can be used to detect the presence of an Ag of interest in the test serum. With the competitive ELISA (cELISA) either a competing Ag or a competing Ab of interest that is present in the test serum will inhibit binding with a known reagent. Both the isELISA and the cELISA can be used when signalling of the Ab of interest is not possible, due to the unavailability of a species-specific secondary Ab (Hamblin et al., 1986, ThermoFisherScientific, 2010, Tizard, 2013).

2.2 Conjugates

These enzyme-labelled secondary antibodies (antiglobulins) are known as *conjugates*, and they work as a detecting mechanism for primary antibodies that are present in the test serum and bound to a specific Ag on the ELISA plate. The Ig-class found most commonly in the serum of healthy animals is IgG and this is also the most common Ig class used as conjugates (Tizard, 2013). Initially the conjugates comprised of a complex mix of all the different classes of antibodies binding with different epitopes at different affinities

(polyclonal) for a particular multivalent Ag, which rendered high sensitivity, but later on monoclonal Ab (MAb) were produced (Kohler and Milstein, 1975) which resulted in a significantly increased specificity of the ELISA. Conjugates prepared from complex mixtures of Ab (polyclonal conjugates) therefore may have highly variable specificity and avidity (combined affinity), and they tend more toward cross-reactivity, whereas MAb conjugates usually bind highly specific with high affinity and are less likely to cross-react with other species (Kindt et al., 2007).

Certain proteins of bacterial origin are able to evade the immune response by binding non-species-specifically with mammalian IgG, mostly in the second and third constant domain of the heavy chain (Kelly et al., 1993), for example protein A (*Staphylococcus aureus*) with 5 binding sites on the Fc, protein G (*Streptococcus*) with 2 binding sites on the Fc, protein L (*Pentostreptococcus magnus*) which bind with the light chain of the IgG at 4 sites, as well as a recombinant fusion protein such as protein A/G, which has 6 known binding sites on the Fc. This ability, which does not inhibit the antigen binding site of the IgG (Feir et al., 1993) makes these proteins useful as conjugates and their respective binding capacities are well-known for humans, as well as most domestic animals and small laboratory mammals (Kelly et al., 1993). The recombinant protein A/G binds strongly with all IgG fractions of cattle, sheep and goats, and the total IgG for horses, donkeys and pigs. protein G also binds strongly to all these species except for pigs, whilst protein A and protein L respectively binds mainly weakly or not at all in these species. For protein L no evidence is available on binding to the equid species (ThermoFisherScientific, 2017). Forsgen and Sjoquist (1966); Kronval et al (1970); Marchalonis et al (1978); Richman et al (1982); Bjorck andKronval (1984); Goudswaard et al. (1978), as cited by Feir (1995) showed

that protein A bound with mammalian IgG of 33 families in 13 orders and protein G with four families in four orders (Feir et al., 1993) with specific affinity, described in studies by Goding, 1978 and Akerström et al., 1985 as cited by Stöbel. It is of particular value when testing mammalian sera with an ELISA as their high affinity for the Fc-region results in higher specificity and very low background staining compared to commercial polyclonal anti-IgG conjugates as shown in studies by Diaz-Aparicio et al., 1994 and Ficapal et al., 1995 as cited by Stöbel (Stöbel et al., 2002).

For the ELISA and other immune-assays these secondary antibody and non-species-specific protein conjugates are labelled with colorimetric enzyme-based detection probes such as alkaline phosphatase (AP) and horseradish peroxidase (HRP). Similarly, fluorescent molecules such as fluorescein (FITC), rhodamine and various other dyes are used to label secondary Ab for immunofluorescent assays and other applications. HRP is a 40 kDa glycoprotein enzyme that reduces hydrogen peroxide in the presence of a variety of possible proton donors (substrates), which produce a measurable colour reaction. HRP is robust with published thermal (up to 60 °C) and pH (4-10) stability, but it is inhibited by azide. HRP substrates include ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]), TMB (3,3',5,5'-tetramethylbenzidine) and OPD (o-phenylenediamine dihydrochloride). OPD can be read at a maximum absorbance of 492 nm after reacting with HRP (Sigma-Aldrich, 2017)[1].

[1] Written permission was granted on 16 Feb 2017 by Sigma-Aldrich to reference the copyright protected information published on their website for this dissertation.

ELISA optimisation is essential for this complex process that is influenced by several factors, for example the enzyme conjugate, for which not only the type of enzyme, the molecule, its binding affinity or avidity and cross-reactivity, as well as the concentration at which it is used are potential causes of failure. It is therefore common practice to optimise two components at a time by checkerboard titration, for example sample concentration and detecting Ab concentration, making sure a strong signal is obtained against low background staining. The recommended starting concentration for coating Ab (purified IgG) optimisation is around 5-15 μg/ml. Checkerboard titration should also be done to determine optimum conjugate concentration and signal detection, ensuring that the Ag (coated IgG) is clearly detectable over a dynamic range. The general recommended secondary Ab concentration for ELISA, using HRP as the enzyme, is 20-200 ng/ml (ThermoFisherScientific, 2010).

2.3 ELISA used for wildlife in the absence of species-specific conjugates

Adapting diagnostic assays developed for domestic animals for use in wildlife is a major process to make provision for variations in the pathogen, exposure, host immune response and cross-reactivity, as well as limitation in terms of reagents used in the laboratory. Such a process should include proper evaluation and characterisation in order to validate the assay and obtain useful test results (Gardner et al., 1996). The absence of species-specific secondary Ab for use in wildlife serology (Abdelgawad et al., 2015) and in particular for the ELISA, is widely recognised and has been the subject of substantial research over the past few decades. This section represents a summary of the studies the author encountered that explored the use of ELISA in wildlife species with a specific focus on African herbivore and hoof-stock wildlife species.

Due to the lack of anti-wildlife conjugates various other methods such as serum neutralisation tests (Barnard and Paweska, 1993, Barnard, 1997, Fischer-Tenhagen et al., 2000, Evans et al., 2008, Lorca-Oró et al., 2014, Abdelgawad et al., 2015), haemagglutination-inhibition (HAI) (Barnard and Paweska, 1993, Fischer-Tenhagen et al., 2000), the complement fixation test (CFT) (Davies et al., 1977, Erasmus, 1978, Fischer-Tenhagen et al., 2000) and the agar gel immunodiffusion (AGID) analyses (Afshar et al., 1987, Feir et al., 1993) were used to serologically confirm exposure to pathogens in African wildlife species towards the end of the twentieth century. As recently as 2004 Turnbull et al. still reported on the assessment of immunity in cheetah and black rhino by injecting mice with their sera to measure passively transferred immunity from anthrax vaccination (Turnbull et al., 2004).

In 1987 Afshar et al. reported on the evaluation of an indirect ELISA (iELISA) as well as a competitive ELISA (cELISA), which they compared to the standard AGID, a modified CFT and the plague neutralisation test to detect antibodies against bluetongue virus in cattle and sheep. In this study they modified the blocking ELISA described by Anderson in 1984, where the antibodies in the test serum compete with a group specific monoclonal bluetongue virus antibody (anti-BTV Ab), thereby demonstrating the presence of antibodies against Bluetongue virus in the test serum and quantifying (titre) it. Together with a publication by Lunt et al. in 1988, it served as the basis for the cELISA currently described in the OIE Terrestrial Manual for testing of this emerging pathogen in wildlife species, using HRP-labelled rabbit anti-mouse IgG conjugate (Afshar et al., 1987, OIE, 2017).

In 1993 Kelly et al. published another significant study that tested protein A and a recombinant protein A/G in a direct ELISA (dELISA) to assess binding with 10 animals from

four laboratory animal species, 10 domestic animal species, including humans, and 16 African wildlife species in Zimbabwe. They observed only weak binding in birds and crocodiles to both conjugates. The horse, goat, buffalo, wildebeest, waterbuck and impala reacted stronger with protein A/G, and this was even more pronounced in elephant, rhinoceros and giraffe. Pig, primates, dog and some small laboratory animals reacted equally well to both conjugates. Their conclusion was that protein A/G is a valuable tool to detect IgG from a wide variety of African wildlife species (Kelly et al., 1993).

Shortly thereafter Feir et al. reported on the assessment of protein A and recombinant protein G in zoo animals using an immunodiffusion assay, and concluded that these molecules may improve the accuracy of certain serological tests for some of the 25 wildlife species they assessed, instead of using secondary Ab from closely related domestic animal species. Testing between 3 and 11 animals per species, they found that the impala, Indian elephant and red kangaroo did not react to either of these molecules in the immunodiffusion assay. Protein A reacted better with black rhinoceros and almost exclusively with carnivores, while sable, kudu and springbok reacted almost solely to protein G (Feir et al., 1993).

Smith et al. then reported in 1995 that an ELISA for horses, using an anti-horse ferritin antibody against the blood-cell protein, had been adapted for use in black and white rhinoceros from unpublished reports (Smith et al., 1995).

In 1996 Lindeque et al. determined vaccine-induced seroconversion in zebra and elephant using a competitive inhibition ELISA described by Turnbull et al. in 1986 to detect antibodies against components of the anthrax toxin in humans and guinea pigs. Lindeque

et al. did, however, not indicate whether they also used the anti-human or anti-guinea pig or another conjugate in these wildlife species (Turnbull et al., 1986, Lindeque et al., 1996).

In that same year Gaborick et al. (1996) described the evaluation of a five-Ag iELISA with a peroxidase protein A and G conjugate to detect bovine tuberculosis in cattle and farmed deer in the USA (Gaborick et al., 1996).

In 2000 Fischer-Tenhage et al. reported on the screening of black- and white-rhinoceros serum for a number of pathogens using several ELISA variations, such as an iELISA and a cELISA for African horse sickness (AHS) described by Hamblin et al. (1992); a cELISA for bluetongue (BT) described by Afshar et al. (1987) and highlighted above; a monoclonal Ab cELISA for epizootic haemorrhagic disease of deer (EHD), described by Thevasagayam et al. (1996) as suitable for use in all animal species; a liquid-phase cELISA for Akabane virus, based on a liquid-phase blocking sandwich ELISA for foot-and-mouth disease described by Hamblin et al. (1986) for cattle; an iELISA and indirect immunofluorescence for RVF Ab, without elaborating on the details of the method; and an immunofluorescence assay for equine herpes virus 1 (EHV-1) Ab, described by Mayr et al. (1977) as cited by Fisher-Tenhage et al. (2000) (Hamblin et al., 1986, Afshar et al., 1987, Fischer-Tenhagen et al., 2000, Thevasagayam et al., 1996).

In 2000 Larson et al. reported on the use of a multiple-Ag ELISA to detect human TB (*Mycobacterium tuberculosis*) in captive elephant in the USA with a protein A and G conjugate mixture (Larsen et al., 2000).

A comprehensive study was published in 2002 when a German-group developed and validated a non-species dependent iELISA to study Lyme disease in zoo animals, using either

non-species-specific conjugated protein A or protein G. Affinity was determined by using a dELISA and they concluded that either of these two commercial conjugates were useful alternatives for 158 of the 160 wildlife species tested, of which 47 species, including primates and pigs, reacted to both conjugates. Although only three animals were tested for most of these species and both intra- and interspecies variation in binding affinity were noted, the reaction within families were homogenous enough to summarise their results per family. Carnivores, camelids, kangaroos and some old-world pigs demonstrated a higher binding affinity for protein A while protein G bound stronger in most ungulate species, confirming the finding of Kelly et al. and Feir et al. (both 1993) 9 years earlier. These include the equids, cervids, bovids as well as rhinoceros and giraffe. The hippopotamus demonstrated intermittent and low binding affinity with protein A and G respectively and only two species of turtle, representing the reptile family, did not react to either conjugate. Most reactions were recorded at serum dilutions of between 1:900 and 1:2700. Other findings of note for specific African species were elephant, which bound to a medium extent only with protein A; zebra, springbok and warthog bound strongly with both protein A and protein G. The rhinoceros species all tended to react stronger to protein G, which is contradictory to the earlier findings of Feir et al. (1993), but supports the finding by Kelly with stronger affinity to protein A/G in these species. Unlike the majority of cloven-hoofed wildlife species who's Ig bound strongly with protein G, blesbok achieved only medium binding, as did kudu, which also bound to a medium extent with protein A, while impala, nyala, gemsbok (oryx), roan and buffalo achieved only weak binding with protein G (Stöbel et al., 2002).

In 2003 Paweska et al. published the evaluation data for an iELISA for RVF using a protein G conjugate, which was tested for several domestic and wildlife herbivore species, including buffalo, eland, kudu and black wildebeest against serum neutralisation and haemagglutination inhibition tests as reference methods (Paweska et al., 2003).

Kramsky et al. (2003) used high-performance liquid chromatography to obtain purified Ig from 11 wildlife species or related species groups and compared their binding affinity to protein G against a positive (cattle) and negative (chicken) control in a dELISA. Seven species performed statistically different from cattle, among which impala and oryx (gemsbok), which reacted significantly less than cattle, whilst the roan/sable and kudu/nyala groups and bontebok bound equivalently (no statistical difference) with protein G, compared to cattle (Kramsky et al., 2003b).

In 2004, due to the unavailability of species-specific conjugates, Turnbull et al. used a goat anti-cat IgG-Fc as a conjugate for an iELISA and a cELISA to determine Ab in cheetah serum for specific components of the anthrax toxin during a vaccine efficiency trail and similarly anti-horse conjugate in the same ELISA on black rhino serum (Turnbull et al., 2004).

Similarly, in 2011 Miller et al. reported using previously published iELISA and cELISA for epizootic haemorrhagic disease (EHD) of deer, bluetongue (BT), Rift valley fever (RVF) and African horse sickness (AHS) in white rhinoceros from serum collected in 2007 (Miller et al., 2011).

In 2013 Pruvot et al. reported on the modification of a commercial *Mycobacterium avium paratuberculosis* (MAP) ELISA kit validated for domestic ruminants to be used for

elk, moose, deer, caribou (reindeer) and bison sera, using anti-bovine, anti-deer and protein G conjugates (Pruvot et al., 2013).

Lorca-Oró et al. (2014) reported on a survey of European wild ruminants, including red, fallow- and roe deer, aodad (Barbary sheep), ibex, mouflon and chamois using a rabbit anti-mouse IgG in a commercial cELISA for BT (OIE, 2017, Lorca-Oró et al., 2014).

Abdelgawad, et al. (2015) used a peptide-based (various glycoproteins) equine herpesvirus (EHV) strain-specific ELISA with purified goat anti-horse IgG for wild equids, which include several species of zebra or protein G for bovidae, such as giraffe, sable, eland and other wildlife species such as rhinoceros and hippopotamus, to study the prevalence of closely related equine herpes viruses in zoo and free-ranging wild animals (Abdelgawad et al., 2015).

2.4 Gaps in literature on ELISA used in wildlife species

The two tables below summarise the current knowledge in terms of non-species-specific protein A, G and A/G conjugates evaluated for binding affinity with IgG of African wildlife species, as well as other innovative ELISA techniques explored due to the lack of species-specific conjugates for wildlife.

Table 2-1 below is a summary of the results of four publications that reported on the use of non-species specific conjugates of whom Kelly, et al. (1993) and Stöbel et al. (2002) made significant and valuable contributions in terms of African wildlife species relevant to this study. The respective units of reporting were also copied in the heading of each column.

Table 2-1. Publications wherein non-species-specific conjugates where tested for use in wildlife

Species	Common name	Kelly et al. (1993) (30 species) OD(±SD)		Stöbel et al. (2002) (160 species) + and - / Feir et al. (1993) (26 spp) No. reacting/no. tested		Kramsky et al. (2003a) (11 species) Relative to Bo curve
		Protein A	Protein A/G	Protein A	Protein G	Protein G
Loxodonta africana	African elephant	0.33(0.14)	1.40(0.29)	++	+	
Equus zebra hartmannae	Hartmann's mountain zebra			+++	+++	
Equus quagga	Burchell's zebra					
Ceratotherium simum	White rhinoceros	0.35(0.17)	2.61(0.11)	-	++	
Diceros bicornis	Black rhinoceros			++	+++	
Phacochoerus africanus	Common warthog			+++	+++	
Hippopotamus amphibius	River hippopotamus			+	+	
Giraffa camelopardalis	Giraffe	0.30(0.03)	2.04(0.38)	-	+++	
Aepyceros melampus	Impala	0.14(0.05)	0.90(0.30)	- 0/7	+ 0/7	Lesser
Alcelaphus buselaphus	Hartebeest					
Connochaetes gnou	Black wildebeest			-	+	
Connochaetes taurinus	Blue wildebeest	0.06(0.01)	1.10(0.34)			
Damaliscus lunatus	Tsessebe					
Damaliscus pygargus phillipsi	Blesbok			-	++	[*D dorcas* equivalent]
Antidorcas marsupialis	Springbok			+++ 1/10	+++ 9/10	
Raphicerus campestris	Steenbok					
Syncerus caffer	African buffalo	0.07(0.01)	2.62(0.22)	-	+	
Tragelaphus angasii	Nyala	0.14(0.05)	1.73(0.35)	-	+	Equivalent (with kudu)
Tragelaphus oryx	Common eland	0.15(0.04)	2.49(0.30)			
Tragelaphus scriptus	Bushbuck	0.24(0.11)	1.66(0.52)			
Tragelaphus strepsiceros	Greater kudu	0.18(0.01)	2.57(0.05)	++	++	Equivalent (with nyala)
Sylvicapra grimmia	Common duiker			[+]	[+]	
Hippotragus equinus	Roan antelope			-	+	Equivalent (with sable)
Hippotragus niger	Sable antelope	0.14(0.04)	2.04(0.48)	- 1/11	+++ 8/11	Equivalent (with roan)
Oryx gazella	Gemsbok			-	+	[*Spp* lesser]
Kobus ellipsiprymnus	Waterbuck	0.08(0.02)	1.2(0.38)	-	+++	
Redunca arundinum	Southern reedbuck			[-]	[+++]	

[] indicates related species in this table.

Table 2-2. Publications where ELISA was used in wildlife research, listing the species, ELISA technique, the conjugates used (if described) and the authors referenced

Common name	ELISA used	Conjugates used	Reference
African elephant (captive)	Multiple Ag-iELISA	Protein A and G conjugate mixture	Larson (2000)
	cELISA (Turnbull, 1986)	Human and /or guinea pig?	Lindeque (1996)
Burchell's zebra	cELISA (Turnbull, 1986)	Human and /or guinea pig?	Lindeque (1996)
	Peptide-based EHV ELISA	Protein G conjugate	Abdelgawad, et.al (2015)
Mountain zebra (Hartmann's)	Peptide-based EHV ELISA	Anti-horse conjugate	Abdelgawad, et.al (2015)
Other wild equids and zebra species	Peptide-based EHV ELISA	Anti-horse conjugate	Abdelgawad, et.al (2015)
White rhinoceros	iELISA and cELISA	Anti-horse conjugate	Miller (2011)
Black rhinoceros	cELISA	Anti-horse conjugate	Turnbull (2004)
White- and black rhinoceros	ELISA developed for equine sera	Anti-horse ferritin antibody	Smith (1995)
White- and black rhinoceros	isELISA and cELISA for AHS, BT, EHD, Akabana virus, an iELISA for RVF	Conjugate not specified Also IFA for EHV (conjugate not determined).	Fisher-Tenhage (2000)
White- and black rhinoceros	peptide based EHV ELISA	Protein G conjugate	Abdelgawad, et.al (2015)
Common warthog			
River hippopotamus	peptide based EHV ELISA	Protein G conjugate	Abdelgawad, et.al (2015)
Giraffe	peptide based EHV ELISA	Protein G conjugate	Abdelgawad, et.al (2015)
Elk, moose, deer, caribou (reindeer)	a modified commercial Mycobacterium avium paratuberculosis (MAP) ELISA kit	anti-Bo, anti-deer and Protein G conjugates	Pruvot (2013)
Red, fallow, roe deer	Antigen-specific cELISA	BTV specific HRP-labelled rabbit anti-mouse IgG	Lorca-Oro (2014) OIE (2017)
Captive cervids	Five-Ag iELISA	Mixture of Protein A and Protein G	Gaborick (1996)
Impala			
Hartebeest			
black wildebeest	iELISA	Protein G	Paweska (2003)
blue wildebeest			
Tsessebe			
Blesbok			
Springbok			
Steenbok			
Bison	A modified commercial Mycobacterium avium paratuberculosis (MAP) ELISA kit	Affinity for anti-bovine, anti-deer and Protein G	Pruvot, 2013)
African buffalo	iELISA	Protein G	Paweska (2003)
Nyala			
Common eland	iELISA	Protein G	Paweska (2003)
	peptide based EHV ELISA	Protein G conjugate	Abdelgawad, et.al (2015)
Bushbuck			
Greater kudu	iELISA	Protein G	Paweska (2003)
Capri: ibex aodad (Barbary sheep)	cELISA assay	BTV specific rabbit anti-mouse IgG	Lorca-Oro (2014)
Ovis: mouflon, chamois	cELISA assay	BTV specific Ab rabbit anti-mouse IgG	Lorca-Oro (2014)
Common duiker			
Roan antelope			
Sable antelope	Peptide-based EHV ELISA	Protein G conjugate	Abdelgawad, et.al (2015)
Gemsbok			
Waterbuck			
Southern reedbuck			

All the other articles found during the literature review process that reported on the use of ELISA techniques in wildlife species, many of which made use of disease-specific conjugates due to the unavailability of species-specific conjugates, are reflected in Table 2-2. This study is an attempt to add to or fill some of the gaps for the exceptional large and diverse indigenous wildlife population of Sub-Saharan Africa (Bengis et al., 2004).

2.5 Measurement of avidity (functional affinity)

Several of the publications discussed in the preceding sections reported differences in the ability and intensity of binding of the conjugate with wildlife IgG and variation between species (inter) and within species (intra) were noted with marked margins of variation in some cases (Kelly et al., 1993, Kramsky et al., 2003b, Stöbel et al., 2002, Pruvot et al., 2013). Stöbel et al. (2002) measured this intra-species variation for two species with 15 and 13 samples respectively and found high individual variation in tigers (± 0.184-0.523) with larger confidence intervals (CI), but relatively low in Przewalski's horses (± 0.098-0.300) with smaller CI, at different serum dilutions. Despite the absence of standardisation of serum protein concentration, which could have contributed to variation in the intensity of reactions in this study, it is significant that interspecies variability did not influence the overall assessment of binding capacity, even at family level (Stöbel et al., 2002).

The bond between antigen and antibody is dynamic, reversible and the total strength of that bond is a result of a combination of non-covalent attractions and repulsions, such as hydrogen and hydrophobic bonds, as well as electromagnetic and Van der Waals' forces (Van Oss et al., 1986). The complementary spatial structure at the binding site influences not only the size of the contact area, but also the closeness of its fit. As some of the binding forces are inversely related to distance, they are highly dependent on the goodness of fit

between the two molecules at the binding site (Bio-Rad, 2017). Antibodies with a better "fit" will therefore bind stronger with the antigen, which introduces the concept of *affinity* as a measure of the strength of the antibody binding to a specific site (hapten) on the antigen. The combined and interdependent strength of the binding between multivalent antibodies with the multiple binding sites of an antigen is, in contrast, referred to as *avidity* (Hudson and Hay, 1989). In the studies discussed in the preceding sections, apart from comparing the binding capacity with that of control species (Pruvot et al., 2013), the avidity of conjugates that bound with African wildlife species were not assessed.

Hudson and Hay (1989) explains the mathematical basis for affinity, but in a heterogeneous (polyclonal) population of different antibody classes there is evidence that the distribution of affinities is often skewed or even bi-modal instead of being random and symmetrically distributed around the mean. Affinity determinations can be done using the effective, but cumbersome standard method of equilibrium dialyses (developed in 1932), whereby the association constant is calculated (Abcam, 2010). Other methods of comparing affinity is to plot the measured optical density (OD) of different conjugates against serum dilution and calculate the area under the curve as a binding affinity index (Pruvot et al., 2013), by transforming data to normality (Larsen et al., 2000) or more expensive technological methods such as bio-specific interaction analyses (Biosensor), which is not always readily assessable (Dauner et al., 2012). Functional antibody affinity (for monoclonal antibodies and single haptens) or avidity (for polyclonal antibodies and/or complex, multivalent antigens) is lately more commonly determined by performing an ELISA assay in the presence and absence of an immune-complex disruptive compound, also called a disassociating or chaotropic agent (Hudson and Hay, 1989, Dimitrov et al., 2011).

This technique is sometimes referred to as the “bind and break ELISA” or the “avidity ELISA” (Dauner et al., 2012).

Chaotropes (CT) provide powerful means to determine the functional relevance of Ab (Dimitrov et al., 2011). These compounds include, urea (with a published range of activity between 1–8 M concentrations); diethylamine (DEA) (range: 1–50 mM); guanidine hydrochloride (GuHCl) (range: 0.5–3.5 M); and thiocyanates, such as potassium or ammonium thiocyanate (NH_4SCN) (range: 0.25–1.75 M) (MacDonald et al., 1988, Dauner et al., 2012).The CT disrupts binding interaction by disturbing the non-covalent hydrogen bonds, hydrophobic and Van der Waals forces between Ab and Ag. Thiocyanates can also affect electrostatic interactions due to their ionic character, making them more universal to study Ab binding avidity. The choatropic effects are time, molarity and temperature dependant. Almanzar et al. (2013) reported specifically on the use of NH4SCN concentration below 3 M at 37°C for 20 minutes (Almanzar et al., 2013). Different authors reported on using different diluents for the CT, for example PBS (Ferreira and Katzin, 1995, Dimitrov et al., 2011) and PBST (0.2% Tween-20) (Dauner et al., 2012). Almanzar et al. (2013) did not mention the diluent used for NH_4SCN.

The aim of using these CT is to break the low avidity or the weaker Ag-Ab bonds. It is, however, essential to consider their disrupting effect on non-covalent protein-interactions of the Ag itself that may impact negatively on the integrity of the protein structure. This is especially relevant if more complex multiprotein Ag are included in an avidity ELISA (Dauner et al., 2012, Dimitrov et al., 2011).

Several statistical methods for calculating the functional affinity or avidity are described, such as plotting the degree of left displacement of the dose-response curve against the $\log_{10}$ serum dilution at 50% reduction (Hudson and Hay, 1989), using a dose-response approach (Ferreira and Katzin, 1995), or using non-linear regression to counter approximations made for linearised regression (Glaser, 1993). Almanzar et al. (2012) expressed the relative avidity index (RAI) as a percentage of the relationship of the measured Ab-concentration with and without the chaotrope. They based their calculations on the virtual absorbance ratio described by Kneitz et al. (2004), who considered this method better than established methods to calculate avidity (Almanzar et al., 2013, Kneitz et al., 2004). Dauner et al. (2012) summarised these calculations for avidity using a chaotrope very succinctly into three groups, namely:

(1) an expression of the molar concentration that causes a 50% reduction in the absorbance signal;
(2) the percentage of the signal that is measured after treatment with the chaotrope; or
(3) the ratio between chaotrope-treated and untreated samples. The published opinion is that the avidity ELISA is an acceptable method and even display superior accuracy (Dauner et al., 2012).

2.6 Factors to consider regarding the use of wildlife serum

The majority of the studies discussed above that reported on the evaluation of conjugates for use in wildlife species, were performed by means of a dELISA, whereby the wildlife serum IgG was coated on the solid phase of the ELISA plate and the conjugates tested were used both as detection and indicator molecules.

The passive adsorption of Ag or Ab to the ELISA plate surface not only depends on time, temperature and pH, but also on the concentration and in particular the protein concentration of the coating solution as it is of particular importance for optimum binding of an Ag or Ab with the surface. Too little Ag or Ab will lead to a very low signal and too much will inhibit binding with the conjugate due to structural (steric) hindrances blocking binding domains (Bio-Rad, 2017, Stöbel et al., 2002). Published recommendations for a dELISA is 20 µg/ml final concentration for Ag (Abcam, 2010) or alternatively 1-10 µg/ml protein concentration, which implies suitable dilution of Ag or Ab for this purpose (Bio-Rad, 2017).

Serum protein reference values for wildlife is scarce, but Feir et al. (1993) reported that measured serum protein concentrations varied between animals of the same species, seemingly without influencing reactivity to both Protein A and G in an immunodiffusion assay (Feir et al., 1993). Stöbel et al (2002) reported that neither total protein nor Ig concentration data were collected for the 160 wildlife species tested and they concluded that binding capacity related to serum dilution instead (Stöbel et al., 2002).

Published serum protein concentration reference values for domestic livestock seems to be somewhat more readily available. Alberghina et al. (2011) published total serum protein concentration determined by biuret reaction as 67.54 ± 11.53 g/ℓ and the protein fractions by electrophoresis including albumin and the different globulin fractions with the albumin/globulin ratio as 0.88 ± 0.43 (Alberghina et al., 2011). It is slightly lower than the reference values determined by electrophoresis by Nagy et al. (2015), who concluded that, despite differences between species on protein fractions, the total protein and IgG concentrations were comparable (Nagy et al., 2015).

In 2013 Alberghina et al. also published reference values, determined similarly to their 2011 study by biuret and electrophoresis, for female donkeys of a specified species as total proteins 50.0-84.0 g/ℓ and the albumin/globulin ratio as 0.41-1.13 (Alberghina et al., 2013).

Serum with its complex, heterogeneous mixed protein composition would be unsuitable as a coating solution for a dELISA, if it was not for the fact that the protein of interest, namely Ig, is so well represented. In the case of such crude Ab preparations it is recommended that a range of concentrations be tested to ensure maximum adsorption and optimum interaction with the conjugate, particularly if accurate protein concentration is not measured or is unknown. (Bio-Rad, 2017).

According to Stöbel et al. (2002) serum dilution was critical and the best binding reactions were obtained within the range of 1:300 to 1:8100 with an optimal dilution of 1:900 to compare the different species, at which 151 of the 160 species reacted with at least one of the non-species-specific conjugates that they used. They ventured an opinion that intra-species differences may be explained by variation in Ig concentration, the presence of binding inhibitors in the sera or possibly to genetic variations, but it did not impact on the outcome of their analyses. To prevent false negative results due to very high protein concentrations, they advised retesting at a higher dilution to confirm such a true negative outcome or rule out insufficient reaction due to binding inhibition (Stöbel et al., 2002). Kelly et al. (1993) reported his findings for 16 African wildlife species at a serum dilution of 1:800 (Kelly et al., 1993).

2.7 Conclusion

There is a clear scope for avidity ELISA analyses to evaluate commercial conjugates for use in African wildlife species and to evaluated intra-species variation.

2.8 References

ABCAM 2010. Understanding secondary antibodies. *In:* ABCAM (ed.).

ABDELGAWAD, A., HERMES, R., DAMIANI, A., LAMGLAIT, B., CZIRJÁK, G. Á., EAST, M., ASCHENBORN, O., WENKER, C., KASEM, S., OSTERRIEDER, N. & GREENWOOD, A. D. 2015. Comprehensive Serology Based on a Peptide ELISA to Assess the Prevalence of Closely Related Equine Herpesviruses in Zoo and Wild Animals. *PLoS ONE,* 10**,** e0138370.

ABUTARBUSH, S. M. 2008. Saunders Comprehensive Veterinary Dictionary, 3rd ed. *The Canadian Veterinary Journal,* 49**,** 906-906.

AFSHAR, A., THOMAS, F. C., WRIGHT, P. F., SHAPIRO, J. L., SHETTIGARA, P. T. & ANDERSON, J. 1987. Comparison of competitive and indirect enzyme-linked immunosorbent assays for detection of bluetongue virus antibodies in serum and whole blood. *J Clin Microbiol,* 25**,** 1705-10.

ALBERGHINA, D., FAZIO, F., ARFUSO, F., SCIANO, S., ZUMBO, A. & PICCIONE, G. 2013. Reference Intervals of Serum Protein Concentrations from Clinically Healthy Female Ragusana Donkeys (Equus asinus) Determined by Cellulose Acetate Electrophoresis. *Journal of Equine Veterinary Science,* 33**,** 433-436.

ALBERGHINA, D., GIANNETTO, C., VAZZANA, I., FERRANTELLI, V. & PICCIONE, G. 2011. Reference intervals for total protein concentration, serum protein fractions, and albumin/globulin ratios in clinically healthy dairy cows. *Journal of Veterinary Diagnostic Investigation,* 23**,** 111-114.

ALLSOPP, B., BABIUK, L. & BABIUK, S. 2004. Vaccination: an approach to the control of infectious diseases. *Infectious Diseases of Livestock***,** 239-247.

ALMANZAR, G., OTTENSMEIER, B., LIESE, J. & PRELOG, M. 2013. Assessment of IgG avidity against pertussis toxin and filamentous hemagglutinin via an adapted enzyme-linked immunosorbent assay (ELISA) using ammonium thiocyanate. *Journal of Immunological Methods,* 387**,** 36-42.

BARNARD, B. J. 1997. Antibodies against some viruses of domestic animals in southern African wild animals. *Onderstepoort J Vet Res,* 64**,** 95-110.

BARNARD, B. J. & PAWESKA, J. T. 1993. Prevalence of antibodies against some equine viruses in zebra (Zebra burchelli) in the Kruger National Park, 1991-1992. *Onderstepoort J Vet Res,* 60**,** 175-9.

BENGIS, R., KOCK, R., THOMSON, G. & BIGALKE, R. 2004. Infectious diseases of animals in sub-Saharan Africa: The wildlife/livestock interface. *Infectious diseases of livestock,* 2.

BIO-RAD. 2017. *Helpful ELISA Hints* [Online]. Online: Bio-Rad. Available: https://www.bio-rad-antibodies.com/helpful-elisa-hints.html [Accessed 14 July 2017 2017].

DAUNER, J. G., PAN, Y., HILDESHEIM, A., KEMP, T. J., PORRAS, C. & PINTO, L. A. 2012. Development and application of a GuHCl-modified ELISA to measure the avidity of anti-HPV L1 VLP antibodies in vaccinated individuals. *Molecular and Cellular Probes,* 26**,** 73-80.

DAVIES, F. G., OTIENO, S. & JESSETT, D. M. 1977. The antibody response in sheep vaccinated with experimental Nairobi sheep disease vaccines. *Tropical Animal Health and Production,* 9**,** 181-183.

DIMITROV, J. D., LACROIX-DESMAZES, S. & KAVERI, S. V. 2011. Important parameters for evaluation of antibody avidity by immunosorbent assay. *Analytical Biochemistry,* 418**,** 149-151.

ERASMUS, B. 1978. A new approach to polyvalent immunization against African horsesickness. *Journal of Equine Medicine and Surgery. Supplement*.

EVANS, A., GAKUYA, F., PAWESKA, J. T., ROSTAL, M., AKOOLO, L., VAN VUREN, P. J., MANYIBE, T., MACHARIA, J. M., KSIAZEK, T. G., FEIKIN, D. R., BREIMAN, R. F. & KARIUKI NJENGA, M. 2008. Prevalence of antibodies against Rift Valley fever virus in Kenyan wildlife. *Epidemiology and infection,* 136**,** 1261-9.

FEIR, D., LAU, C. & JUNGE, R. 1993. Protein A and protein G in the diagnosis of diseases in zoo animals. *Transactions of the Missouri Academy of Science,* 27**,** 9-14.

FERREIRA, M. U. & KATZIN, A. M. 1995. The assessment of antibody affinity distribution by thiocyanate elution: a simple dose-response approach. *Journal of Immunological Methods,* 187**,** 297-305.

FISCHER-TENHAGEN, C., HAMBLIN, C., QUANDT, S. & FROLICH, K. 2000. Serosurvey for selected infectious disease agents in free-ranging black and white rhinoceros in Africa. *J Wildl Dis,* 36**,** 316-23.

GABORICK, C. M., SALMAN, M. D., ELLIS, R. P. & TRIANTIS, J. 1996. Evaluation of a five-antigen ELISA for diagnosis of tuberculosis in cattle and Cervidae. *J Am Vet Med Assoc,* 209**,** 962-6.

GARDNER, I. A., HIETALA, S. & BOYCE, W. M. 1996. Validity of using serological tests for diagnosis of diseases in wild animals. *Rev Sci Tech,* 15.

GLASER, R. W. 1993. Determination of antibody affinity by ELISA with a non-linear regression program: Evaluation of linearized approximations. *Journal of Immunological Methods,* 160**,** 129-133.

HAJELA, K. 1991. Structure and function of Fc receptors. *Biochemical education,* 19**,** 50-57.

HAMBLIN, C., BURNETT, I. T. R. & HEDGER, R. S. 1986. A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus. I. Development and method of ELISA. *Journal of Immunological Methods,* 93**,** 115-121.

HUDSON, L. & HAY, F. C. 1989. *Practical Immunology,* Great Britain, The Bath Press.

JENNER, E. 1800. An inquiry into the causes and effects of the variolae vaccinae, a disease discovered in some of the western counties of England, particularly Gloucestershire, and known by the name of the cow pox, author.

JOHNSON, M. 2012. Secondary Antibody Review. *Labome Materials and Methods,* 2.

KELLY, P. J., TAGWIRA, M., MATTHEWMAN, L., MASON, P. R. & WRIGHT, E. P. 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.

KINDT, T. J., GOLDSBY, R. A. & OSBORNE, B. A. 2007. Kuby immunology. New York: WH Freeman.

KNEITZ, R.-H., SCHUBERT, J., TOLLMANN, F., ZENS, W., HEDMAN, K. & WEISSBRICH, B. 2004. A new method for determination of varicella-zoster virus immunoglobulin G avidity in serum and cerebrospinal fluid. *BMC infectious diseases,* 4**,** 33.

KOHLER, G. & MILSTEIN, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature,* 256**,** 495-497.

KRAMSKY, J. A., MANNING, E. J. B. & COLLINS, M. T. 2003. Protein G binding to enriched serum immunoglobulin from nondomestic hoofstock species. *J Vet Diagn Invest,* 15.

LARSEN, R. S., SALMAN, M. D., MIKOTA, S. K., ISAZA, R., MONTALI, R. J. & TRIANTIS, J. 2000. Evaluation of a multiple-antigen enzyme-linked immunosorbent assay for detection of Mycobacterium tuberculosis infection in captive elephants. *J Zoo Wildl Med,* 31**,** 291-302.

LEQUIN, R. M. 2005. Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). *Clin Chem,* 51**,** 2415-8.

LINDEQUE, P., BRAIN, C., VERSFELD, W. & TURNBULL, P. 1996. Anthrax vaccine-induced seroconversion in zebra and elephant in the Etosha National Park, Namibia. *Salisbury Med Bull,* 87**,** 113-5.

LIPMAN, N. S., JACKSON, L. R., TRUDEL, L. J. & WEIS-GARCIA, F. 2005. Monoclonal Versus Polyclonal Antibodies: Distinguishing Characteristics, Applications, and Information Resources. *ILAR Journal,* 46**,** 258-268.

LORCA-ORÓ, C., LÓPEZ-OLVERA, J. R., RUIZ-FONS, F., ACEVEDO, P., GARCÍA-BOCANEGRA, I., OLEAGA, Á., GORTÁZAR, C. & PUJOLS, J. 2014. Long-Term Dynamics of Bluetongue Virus in Wild Ruminants: Relationship with Outbreaks in Livestock in Spain, 2006-2011. *PLOS ONE,* 9**,** e100027.

MACDONALD, R. A., HOSKING, C. S. & JONES, C. L. 1988. The measurement of relative antibody affinity by ELISA using thiocyanate elution. *Journal of Immunological Methods,* 106**,** 191-194.

MILLER, M., BUSS, P., JOUBERT, J., MASEKO, N., HOFMEYR, M. & GERDES, T. 2011. Serosurvey for selected viral agents in white rhinoceros (Ceratotherium simum) in Kruger National Park, 2007. *J Zoo Wildl Med,* 42**,** 29-32.

NAGY, O., TOTHOVA, C., NAGYOVA, V. & KOVAC, G. 2015. Comparison of serum protein electrophoretic pattern in cows and small ruminants. *Acta Veterinaria Brno,* 84**,** 187-195.

NOBEL, M. 2014. *The Nobel Prize in Physiology or Medicine 1984* [Online]. Nobelprize.org: Nobel Media AB. Available: http://www.nobelprize.org/nobel_prizes/medicine/laureates/1984/ [Accessed 15 Feb 2017].

OIE 2017. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2017. Online: OIE.

PAWESKA, J. T., SMITH, S. J., WRIGHT, I. M., WILLIAMS, R., COHEN, A. S., VAN DIJK, A. A., GROBBELAAR, A. A., CROFT, J. E., SWANEPOEL, R. & GERDES, G. H. 2003. Indirect enzyme-linked immunosorbent assay for the detection of antibody against Rift Valley fever virus in domestic and wild ruminant sera. *The Onderstepoort journal of veterinary research,* 70**,** 49-64.

PRUVOT, M., FORDE, T. L., STEELE, J., KUTZ, S. J., BUCK, J. D., MEER, F. V. D. & ORSEL, K. 2013. The modification and evaluation of an ELISA test for the surveillance of Mycobacterium avium subsp. paratuberculosisinfection in wild ruminants. *BMC Veterinary Research,* 9**,** 5.

SIGMA-ALDRICH. 2017. *Secondary Antibodies, Conjugates and Kits* [Online]. http://www.sigmaaldrich.com: Sigma-Aldrich. Available: http://www.sigmaaldrich.com [Accessed 16 February 2017 2017].

SMITH, J. E., CHAVEY, P. S. & MILLER, R. E. 1995. Iron Metabolism in Captive Black (Diceros bicornis) and White (Ceratotherium simum) Rhinoceroses. *Journal of Zoo and Wildlife Medicine,* 26**,** 525-531.

STÖBEL, K., SCHONBERG, A. & STAAK, C. 2002. A new non-species dependent ELISA for detection of antibodies to Borrelia burgdorferi s. l. in zoo animals. *Int J Med Microbiol,* 291 Suppl 33**,** 88-99.

THERMOFISHERSCIENTIFIC 2010. ELISA technical guide and protocols. *In:* INCORPORATE, T. F. S. (ed.). Online: Thermo Fisher Scientific Incorporate.

THERMOFISHERSCIENTIFIC. 2017. *Comparison of Antibody IGg Binding Proteins* [Online]. Antibody Resource Library: ThermoFisher Scientific. Available: https://www.thermofisher.com/za/en/home/life-science/antibodies/antibodies-learning-center/antibodies-resource-library/antibody-methods/comparison-antibody-igg-binding-proteins.html [Accessed 6 July 2017 2017].

THEVASAGAYAM, J. A., WOOLHOUSE, T. R., MERTENS, P. P., BURROUGHS, J. N. & ANDERSON, J. 1996. Monoclonal antibody based competitive ELISA for the detection of antibodies against epizootic haemorrhagic disease of deer virus. *J Virol Methods,* 57**,** 117-26.

THRUSFIELD, M. 2013. *Veterinary epidemiology,* Great Brittain, Elsevier.

TIZARD, I. R. 2013. *Veterinary immunology,* St. Louis, Mo. :, Elsevier/Saunders.

TURNBULL, P. C. B., BROSTER, M. G., CARMAN, J. A., MANCHEE, R. J. & MELLING, J. 1986. Development of antibodies to protective antigen and lethal factor components of anthrax toxin in humans and guinea pigs and their relevance to protective immunity. *Infection and Immunity,* 52**,** 356-363.

TURNBULL, P. C. B., TINDALL, B. W., COETZEE, J. D., CONRADIE, C. M., BULL, R. L., LINDEQUE, P. M. & HUEBSCHLE, O. J. B. 2004. Vaccine-induced protection against anthrax in cheetah (Acinonyx jubatus) and black rhinoceros (Diceros bicornis). *Vaccine,* 22**,** 3340-3347.

VAN OSS, C., GOOD, R. & CHAUDHURY, M. 1986. Nature of the antigen-antibody interaction: Primary and secondary bonds: optimal conditions for association and dissociation. *Journal of Chromatography B: Biomedical Sciences and Applications,* 376**,** 111-119.

Chapter 3 Materials and methods

3.1 Approvals and consent

Approvals obtained to conduct the study included animal ethics approval by the University of Pretoria Animal Ethics Committee with project approval number: V086-16, granted on 25 July 2016 and extended on 15 March 2017 for the duration of 2017. In addition to this, approval for the use of animal material in research was granted in terms Section 20 of the Animal Diseases Act (Act 35 of 1984) on 1 August 2016 with ref no: SDAH-Epi-16080206460 and amended on 17 February 2017 with ref. no: SDAH-Epi-17022010481.

Informed consent was obtained from four suppliers of stored wildlife serum, namely Dr Pierre Nel of the Free State Department of Small Business, Economic Development, Tourism and Environmental Affairs, Bloemfontein; Dr Peter Buss of the Wildlife Veterinary Services of the South African National Parks, Skukuza; Dr AP Malgraff of the Western Cape Department of Agriculture, Export Control, Mosstrich, Mossel Bay; and the Western Cape Department of Agriculture, Animal Health State Veterinary Office: Beaufort-West.

3.2 Conjugates

Purified recombinant protein A/G: HRP (ThermoFisher Scientific) sourced from LTC Tech South Africa (Pty) Ltd. and two polyclonal horse radish peroxidase (HRP) conjugates commercially available and sourced from local suppliers in South Africa, namely: rabbit anti-bovine IgG (H/L): HRP (Bio-Rad Laboratories Inc.), which binds with both the heavy and light chains of bovine IgG from Celtic Molecular Diagnostics, South Africa; and goat anti-

horse IgG (Fc specific): HRP IgG (Sigma-Aldrich (Pty.) Ltd.) which binds only with the Fc-fraction of IgG

from Merck (Pty.) Ltd., South Africa. In the rest of this thesis these conjugates are called protein A/G: HRP, anti-bovine IgG: HRP and anti-horse IgG: HRP.

3.3 Serum (coating antigen)

No targeted serum collection was necessary for this project as stored or remaining serum of animals bled for other purposes such as disease surveillance, translocation, research, genetic resource purposes and animals bled after hunting for export purposes were sourced and live animals were therefore not specifically immobilised and bled for this project.

Stored serum of 27 common South African herbivore and hoof stock wildlife species, of which 20 species are classified as ruminants, were obtained. These species are listed in Table 3-1 below according to their taxonomic classification (Skinner and Chimimba, 2005).

Cattle (bovine) serum was used as positive control for the protein A/G: HRP and anti-bovine IgG: HRP and horse (equine) serum was used as positive control for the anti-horse IgG: HRP. Both control sera were sourced from the serum bank of the serology laboratory of the Department of Veterinary Tropical Diseases at the Faculty of Veterinary Science, Onderstepoort.

Table 3-1: List of 27 wildlife species for which serum was obtained

Order	Family	Subfamily	Species	Common name
Proboscidae	Elephantidae		*Loxodonta africana*	African elephant
Perissodactyla (odd-toed)	Equidae		*Equus zebra hartmannae*	Hartmann's mountain zebra
			Equus quagga	Burchell's zebra
	Rhinocerotidae		*Ceratotherium simum*	White rhinoceros
			Diceros bicornis	Black rhinoceros
Artiodactyla (even-toed)	Suidae		*Phacochoerus africanus*	Common warthog
	Hippopotamidae		*Hippopotamus amphibius*	River hippopotamus
Sub-order Ruminantia				
	Giraffidae		*Giraffa camelopardalis*	Giraffe
	Bovidae	Aepycerotinae	*Aepyceros melampus*	Impala
		Alcelaphinae	*Alcelaphus buselaphus*	Hartebeest
			Connochaetes gnou	Black wildebeest
			Connochaetes taurinus	Blue wildebeest
			Damaliscus lunatus	Tsessebe
			Damaliscus pygargus phillipsi	Blesbok
		Antilopinae	*Antidorcas marsupialis*	Springbok
			Raphicerus campestris	Steenbok
		Bovinae	*Syncerus caffer*	African buffalo
			Tragelaphus angasii	Nyala
			Tragelaphus oryx	Common eland
			Tragelaphus scriptus	Bushbuck
			Tragelaphus strepsiceros	Greater kudu
		Cephalophinae	*Sylvicapra grimmia*	Common duiker
		Hippotraginae	*Hippotragus equinus*	Roan antelope
			Hippotragus niger	Sable antelope
			Oryx gazella	Gemsbok
		Reduncinae	*Kobus ellipsiprymnus*	Waterbuck
			Redunca arundinum	Southern reedbuck

3.4 Chaotrope

Ammonium thiocyanate (NH_4SCN) was used as a chaotropic agent and sourced from the chemical store of the Department of Veterinary Tropical Diseases of the Faculty of Veterinary Science of the University of Pretoria. NH_4SCN is an inorganic compound with a molar weight of 71,12 g. The appropriate molarity of the chaotrope required to break the weak bonds between the primary Ab and the anti-species conjugates, as well as the effect of the chaotrope on the reactivity of the conjugated HRP enzyme (Sigma-Aldrich, 2017) had to be evaluated.

3.5 Determine the effect of the chaotrope on the conjugate and the coating buffer

To determine the effect of different molarities of the chaotrope (CT) and two different coating buffers on the conjugate enzyme reactivity, a simple direct ELISA (dELISA) was performed by coating the conjugate directly to the plate. All ELISAs were performed using Nunc MaxiSorp™ 96 well ELISA plates (ThermoFisher Scientific). Fifty microlitres (protein A/G: HRP conjugate at 1:1000 dilution in 0,05 M carbonate/bicarbonate buffer (CB) of pH 9,6 were added to all wells in columns 1, 2, 3 and 4. The same was added to columns 7, 8, 9 and 10 but using 0,15 M phosphate-buffered saline (PBS) pH 7,2 instead of CB.

After 45 minutes incubation at 37°C on an orbital shaker, plates were washed three times with PBS supplemented with 0.05% Tween 20 (PBST) by filling and emptying the wells. Fifty microlitres of NH_4SCN at 2 M, 1,75 M, 1,5 M, 1,25 M, 1 M, 0,75 M, 0,5 M or 0,25 M (in distilled water) were added in duplicate wells in columns 1 and 2 as well as columns 7 and 8. Positive control wells in columns 3, 4, 9 and 10 received 50 µl of PBS. A schematic representation of the layout of this specific dELISA plate is available below as Figure 3-1.

The plate was again incubated and washed as before. Fifty microlitres of freshly prepared o-phenylenediamine dihydrochloride (OPD) (Sigma) at 0,4 mg/ml in distilled water plus 0,05% hydrogen peroxide (30%) was added as chromogen and substrate to all wells. Colour development was blocked after 5 minutes by the addition of 50 µl of 1 M sulphuric acid (H_2SO_4). The optical density was read in an ELISA reader, using the Gen5 software (Analytical and Diagnostic Products) at a wavelength of 490 nm.

	1	2	3	4	5	6	7	8	9	10	11	12
A	2 M	2 M	PBS	PBS			2 M	2 M	PBS	PBS		
B	1.75 M	1.75 M	PBS	PBS			1.75 M	1.75 M	PBS	PBS		
C	1.5 M	1.5 M	PBS	PBS			1.5 M	1.5 M	PBS	PBS		
D	1.25 M	1.25 M	PBS	PBS			1.25 M	1.25 M	PBS	PBS		
E	1 M	1 M	PBS	PBS			1 M	1 M	PBS	PBS		
F	0.75 M	0.75 M	PBS	PBS			0.75 M	0.75 M	PBS	PBS		
G	0.5 M	0.5 M	PBS	PBS			0.5 M	0.5 M	PBS	PBS		
H	0.25 M	0.25 M	PBS	PBS			0.25 M	0.25 M	PBS	PBS		

Figure 3-1: Schematic description of the plate layout for the dELISA to determine the effect of the chaotrope (CT) and two different coating buffers on the conjugate. Protein A/G in carbonate/bicarbonate buffer (yellow shading) was coated to columns 1-4 and protein A/G in PBS (pink shading) to columns 7-10. NH_4SCN was titrated at different molar concentration in columns 1, 2, 7 and 8 while columns 3, 4, 9 and 10 received PBS as control treatment.

3.6 Determine optimum reagent solution

3.6.1 Checker-board titration to determine serum and conjugate dilution

To determine the binding capacity of different enzyme conjugates to various animal sera, a direct Elisa setup was used where the plates were coated with serum from a specific species and then incubated with a specific enzyme conjugate. To determine optimum serum dilution for coating plates checker-board titrations were performed with bovine serum for both the protein A/G: HRP and the anti-bovine IgG: HRP and with equine serum for the anti-horse IgG: HRP.

A two-fold serial serum dilution, from 1:125 to 1:8000, was constituted in PBS in seven replicate wells (50 µl/well) in rows A-G across all 12 columns of the ELISA plate. Row H served as a negative control with 50 µl PBS in the 12 wells. The plates were incubated in a humid chamber for 1 hour at 37°C on an orbital shaker. After washing with distilled water three times by filling and emptying the wells, all wells were filled with 200 µl of PBST

supplemented with 5% milk powder and incubated for 30 minutes at 37°C on an orbital shaker in a blocking step. The wells were emptied properly by firmly shaking out all content.

A two-fold serial conjugate dilution was constituted in PBST supplemented with 5% milk powder in eleven replicate wells (50 µl/well) in columns 1-11 across all 8 rows of the ELISA plate, with column 12 as the negative control wells. The dilution series started from 1:5000 for protein A/G: HRP and from 1:1000 for the anti-bovine IgG: HRP and anti-horse IgG: HRP. The plates were again incubated in a humid chamber for 1 hour at 37°C on an orbital shaker, wash four times with distilled water by filling and emptying of the wells and then soaked for 1 minute after filling all wells with PBST. The wells were emptied properly by firmly shaking out all content.

The same substrate and chromogen method as described above was used throughout the project and 50 µl OPD (Sigma) at 0,4 mg/ml in distilled water plus 0,05% hydrogen peroxide (H_2O_2) (30%), were added to all wells. Colour development was blocked after 12 minutes by the addition of 50 µl 1 M sulphuric acid (H_2SO_4). The optical density was read in an ELISA reader, using the Gen5 software (Analytical and Diagnostic Products) at a wavelength of 490 nm.

3.6.2 Titration to determine optimum chaotrope concentration

To determine a relative avidity index (RAI) for the binding between antibody from the various species and the different anti-species conjugates, a ratio between the colour development of CT-treated and non-treated wells was calculated. A CT concentration that resulted in less than 50% reduction in binding of the conjugates to the bovine serum antibodies was needed. This was determined in separate ELISA plates prepared for the

protein A/G: HRP and anti-bovine IgG: HRP, coated with bovine serum. After addition of the conjugates, the CT and control treatment was applied at 0,25 M incremental CT concentrations. The effect of the different CT concentrations on the binding of both conjugates were measured (opacity density) and the ratio of CT-treated to untreated samples were calculated.

The same ELISA conditions were used as described in section 4.3 below. Briefly: the serum was coated at 1:2000. The chosen conjugates dilutions of 1:10000 for protein A/G: HRP and 1:4000 for the anti-bovine IgG: HRP was added to all wells. Due to the dilution factor of the CT or PBS added as treatments into all wells, the conjugates were prepared at half the required dilution (double concentration) in PBTS + 10% milk powder (double concentration) to ensure correct end-concentrations in each well. This principle was followed throughout the rest of the project where similar circumstances required it. After a blocking step the NH_4SCN at a final concentration of 2 M, 1,75 M, 1,5 M, 1,25 M, 1 M, 0,75 M, 0,5 M and 0,25 M were added in duplicate wells for each plate and the corresponding duplicate wells received PBS instead. After colour development the percent reduction in colour in the CT-treated wells as compared to the PBS-treated wells was recoded.

3.7 Experimental design

This study was designed to identify commercially available cross-reactive conjugates that can potentially be used in ELISA assays for various wildlife species. Conjugates were therefore selected to ensure an increased likelihood of cross-reactivity or binding to wildlife serum IgG. In addition to the above, the study design provided the opportunity to evaluate

the binding avidity of these bonds in order to quantify the usefulness of promising conjugates.

This is a fundamental experimental study. Despite some reported intraspecies variation in cross-reactivity (Kelly et al., 1993, Kramsky et al., 2003b, Stöbel et al., 2002, Pruvot et al., 2013), intraspecies binding variability was unknown for many species. Large variation may, however, be prone to wider confidence intervals (CI), which may render the data statistically vulnerable. The sample size was, therefore, based on the estimated confidence intervals (CI), calculated from the proportion of positive reactions per sample size. The estimated CI for three different sample sizes are illustrated in Figure 3-2 below, which demonstrates the variation in the CI with variation in the proportion of antibody-antigen binding compared to an assumed 100% binding in the true target (control) species.

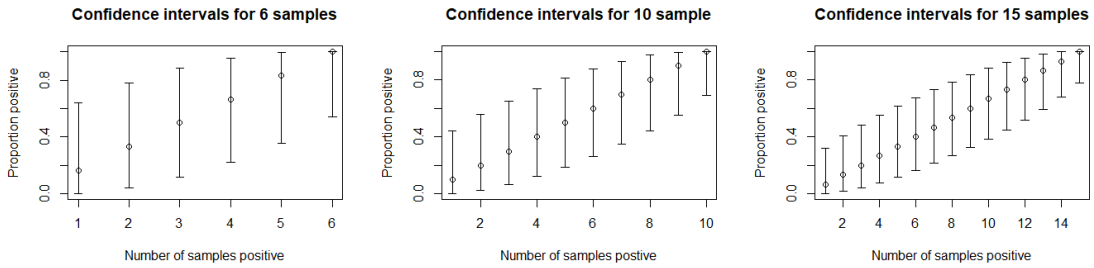

Figure 3-2: Estimated confidence interval relating to the number of samples and the proportion of Ab-Antigen binding. These graphs were plotted using the prevalence package propCI-function in R.

A number of at least 10 samples per species was desirable, tested in duplicate at a single dilution. Ten serum samples were subsequently tested for each species except where otherwise indicated. This sample size was also used by Kelly et al. (1993).

After completion of optimisation studies for all reagents and conditions as described above a simple dELISA was implemented. The ELISA plate structure was designed to accommodate two species per plate with two sets of duplicate wells (CT treatment and PBS

control) for serum samples of each of the 10 animals as illustrated in Figure 3-3 below. The positive control was also coated in duplicate for every row in columns 11 and 12. Three identical plates were coated, one for each of the three selected conjugates, namely the recombinant protein A/G: HRP, the rabbit anti-bovine IgG: HRP and the goat anti-horse IgG: HRP.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6	7	8	9	10	+C	+C
B	1	2	3	4	5	6	7	8	9	10	+C	+C
C	1	2	3	4	5	6	7	8	9	10	+C	+C
D	1	2	3	4	5	6	7	8	9	10	+C	+C
E	1	2	3	4	5	6	7	8	9	10	+C	+C
F	1	2	3	4	5	6	7	8	9	10	+C	+C
G	1	2	3	4	5	6	7	8	9	10	+C	+C
H	1	2	3	4	5	6	7	8	9	10	+C	+C

Figure 3-3: ELISA plate structure for the evaluation of the binding capacity of enzyme conjugates in different wildlife species. Wells A1 to D10 were coated with serum from one species and wells E1 to H10 were used for another species. Either bovine or equine serum was coated in columns 11 and 12. PBS was added to wells in rows A, B, E and F while the chaotrope was added to rows C, D, G and H.

3.8 Experimental procedure

To test cross-reactivity and functional avidity for three commercial conjugates with 27 wildlife species serum IgG using a dELISA plates were coated with 10 serum samples per species at the selected serum dilution of 1:2000. As illustrated in Figure 3-3 above, three identical plates were coated with two species per plate and a positive control for each of the conjugates. Two plates were coated with bovine serum as control while the other one was coated with equine serum. Each enzyme conjugate was tested on a separate plate.

Plates were coated by dispensing 50 µl of serum diluted in PBS in the wells of rows A-D of the first column and repeating the procedures for each of the ten individual wildlife serum samples for that species up to column 10. The diluted serum of the ten samples of

the second species was similarly dispensed into rows E-H of columns 1-10 and 50 µl 1:2000 diluted control serum was added to all wells in columns 11-12.

After 60 minutes at 37°C incubation on an orbital shaker, plates were washed three times with distilled water by filling and emptying the wells. All wells were subsequently filled with 200 µl of PBST supplemented with 5% milk powder and incubated for 30 minutes at 37°C on an orbital shaker as a blocking step. The wells were emptied properly by firmly shaking out all content.

Fifty microlitres of the conjugate, diluted with PBST + 5 % milk powder, at a final dilution in the well of 1:10000 for protein A/G: HRP, 1:2000 for the anti-bovine IgG: HRP and 1:4000 for the anti-horse IgG: HRP, were added to all wells of the respective plates. Fifty microliter PBS was dispensed into all wells in rows A, B, E and F, while 50 µl of the CT at a final concentration of 0,25 M in the well, were subsequently added to all wells in rows C, D, G and H.

The plates were again incubated for 60 minutes at 37°C on an orbital shaker and washed four times with distilled water by filling and emptying of the wells and then soaked for 1 minute after filling all wells with PBST. The wells were emptied properly by firmly shaking out all content.

The Ab-conjugate binding was visualised by adding 50 µl of freshly prepared OPD (Sigma) at 0,4 mg/ml in distilled water plus 0,05% hydrogen peroxide (30%) as chromogen and substrate to all wells. Colour development was blocked after 12 minutes by the addition of 50 µl of 1 M sulphuric acid (H_2SO_4). The optical density was read in an ELISA

reader, using the Gen5 software (Analytical and Diagnostic Products) at a wavelength of 490 nm.

3.9 Data and statistical analyses

The results were captured in Excel and data were assessed using normal mathematical calculations, including division, subtraction, multiplication and summation. Further analysed were performed in Excel and R, calculating mean, standard deviation, variance and the percent reduction in colour in the CT-treated compared to the PBS-treated wells. A cut-off value for positive reactions were set at 50% of the OD value of the control species for the PBS-treated wells on each plate. Intraspecies variation was calculated for all test samples as the standard deviation (variance).

In order to compare differences in cross-reactivity of the conjugates between the species, the OD values obtained for each specie's serum and enzyme conjugate were normalised to a control bovine or equine serum that was included for each species on the same assay plate. This was done by subtracting the OD values obtained for the standard serums from the ODs of the test serums, effectively setting the baseline for the standard serum at zero and positive or negative differences indicating more or less binding of the conjugate relative to the standard.

Further analyses compared the mean OD (difference in binding) of the tested serum with the control serum by means of a two-sided t-test. The Welch Two Sample t-test was the statistical test used to compare the difference of the means of the test and control samples and the t-value, the p-value, the variance and the 95% confidence intervals, which confirms significance if it does not contain a zero, was calculated for each conjugate using

R version 3.3.3 (2017-03-06) - "Another Canoe" Copyright (C) 2017. The R Foundation for Statistical Computing Platform: x86_64-w64-mingw32/x64 (64-bit), with the R-package Rmisc. Frequency distributions for the mean PBS-treated OD values were calculated by using the histogram function in Excel and the hist-function in R. The quantiles for the mean OD values for the PBS treatment of the wildlife species were calculated using the quantile-function in R.

The relative avidity index was calculated as the ratio of the OD of CT-treated /control (PBS-treated) wells for each species and conjugate. Both the sum and product of the normalised OD and the RAI were calculated and proposed as usefulness indexes.

The student completed all the calculations and analyses personally. She submitted the results for advice to Prof Dirk Berkvens (ITM) who indicated that the methods followed seemed sufficient and valid considering the fundamental nature of this study.

3.10 References

DAUNER, J. G., PAN, Y., HILDESHEIM, A., KEMP, T. J., PORRAS, C. & PINTO, L. A. 2012. Development and application of a GuHCl-modified ELISA to measure the avidity of anti-HPV L1 VLP antibodies in vaccinated individuals. *Molecular and Cellular Probes,* 26**,** 73-80.

DIMITROV, J. D., LACROIX-DESMAZES, S. & KAVERI, S. V. 2011. Important parameters for evaluation of antibody avidity by immunosorbent assay. *Analytical Biochemistry,* 418**,** 149-151.

FERREIRA, M. U. & KATZIN, A. M. 1995. The assessment of antibody affinity distribution by thiocyanate elution: a simple dose-response approach. *Journal of Immunological Methods,* 187**,** 297-305.

KELLY, P. J., TAGWIRA, M., MATTHEWMAN, L., MASON, P. R. & WRIGHT, E. P. 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.

KRAMSKY, J. A., MANNING, E. J. B. & COLLINS, M. T. 2003. Protein G binding to enriched serum immunoglobulin from nondomestic hoofstock species. *J Vet Diagn Invest,* 15.

PRUVOT, M., FORDE, T. L., STEELE, J., KUTZ, S. J., BUCK, J. D., MEER, F. V. D. & ORSEL, K. 2013. The modification and evaluation of an ELISA test for the surveillance of Mycobacterium avium subsp. paratuberculosisinfection in wild ruminants. *BMC Veterinary Research,* 9**,** 5.

SIGMA-ALDRICH. 2017. *Secondary Antibodies, Conjugates and Kits* [Online]. http://www.sigmaaldrich.com: Sigma-Aldrich. Available: http://www.sigmaaldrich.com [Accessed 16 February 2017 2017].

SKINNER, J. D. & CHIMIMBA, C. T. 2005. *The Mammals of the Southern African Sub-region*, Cambridge University Press.

STÖBEL, K., SCHONBERG, A. & STAAK, C. 2002. A new non-species dependent ELISA for detection of antibodies to Borrelia burgdorferi s. l. in zoo animals. *Int J Med Microbiol,* 291 Suppl 33**,** 88-99.

THRUSFIELD, M. 2013. *Veterinary epidemiology*, Elsevier.

TURNBULL, P. C. B., TINDALL, B. W., COETZEE, J. D., CONRADIE, C. M., BULL, R. L., LINDEQUE, P. M. & HUEBSCHLE, O. J. B. 2004. Vaccine-induced protection against anthrax in cheetah (Acinonyx jubatus) and black rhinoceros (Diceros bicornis). *Vaccine,* 22**,** 3340-3347.

Chapter 4 Results

4.1 The effect of the chaotropic agent and coating buffers on the reactivity of the enzyme conjugate

A dELISA was used to evaluate the effect of a chaotropic agent, ammonium thiocyanate (NH_4SCN) and two different coating buffers *vs* CB and PBS, on the reactivity of the enzyme conjugate. Mean OD values obtained for the wells with and without CT and that were coated with CB and PBS respectively are presented in Figure 4-1 andFigure 4-2: The effect of the chaotrope (CT) and the PBS coating buffer on the reactivity of the conjugate enzyme. There was no decrease in reactivity in the wells that were coated in PBS and treated with the CT.Figure 4-2.

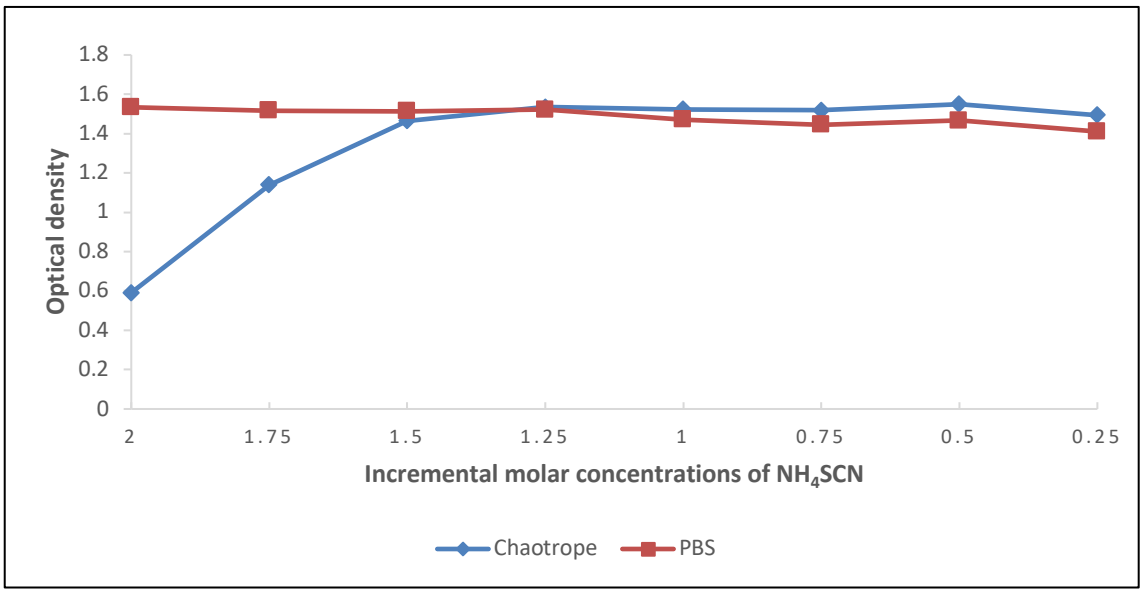

Figure 4-1: The effect of the chaotrope (CT) and the carbonate/bicarbonate coating buffer (CB) on the reactivity of the conjugate enzyme. Wells coated with CB and treated with the CT at 2 M and 1,75 M concentrations showed a decrease in conjugate enzyme reactivity.

Wells coated with CB and treated with the CT at 2 M and 1,75 M concentrations showed a decrease in conjugate enzyme reactivity. There was no decrease in reactivity in the wells that were coated in PBS and treated with CT.

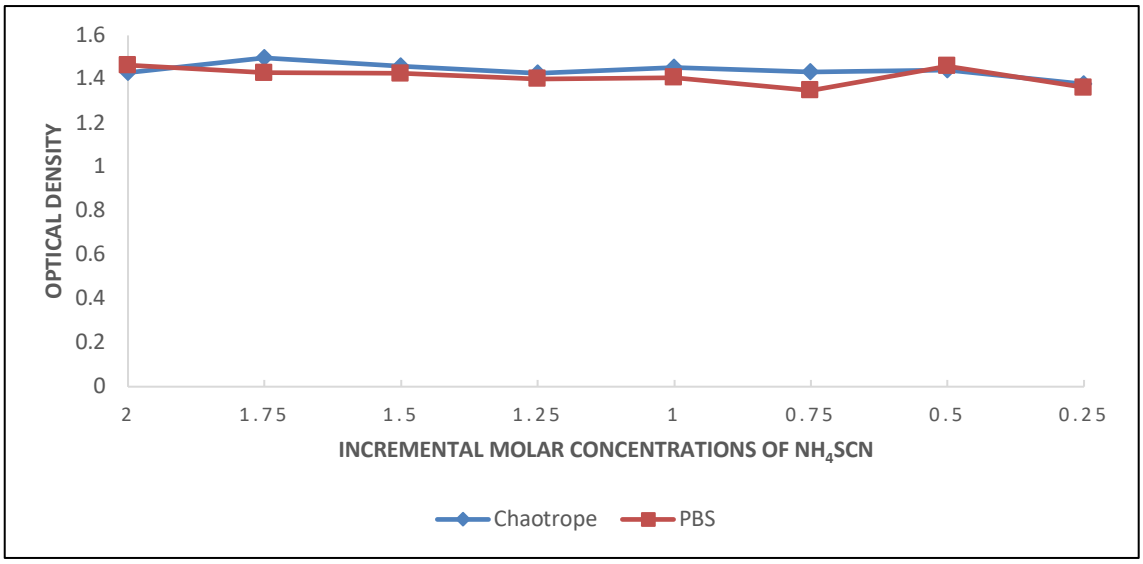

Figure 4-2: The effect of the chaotrope (CT) and the PBS coating buffer on the reactivity of the conjugate enzyme. There was no decrease in reactivity in the wells that were coated in PBS and treated with the CT.

4.2 Optimisation of reagent concentrations

4.2.1 Checker-board titration to determine the optimum serum and conjugate dilution

To determine optimum serum and conjugate dilutions a checker-board titration was performed with bovine serum and protein A/G: HRP. The results are demonstrated in Figure 4-3 below.

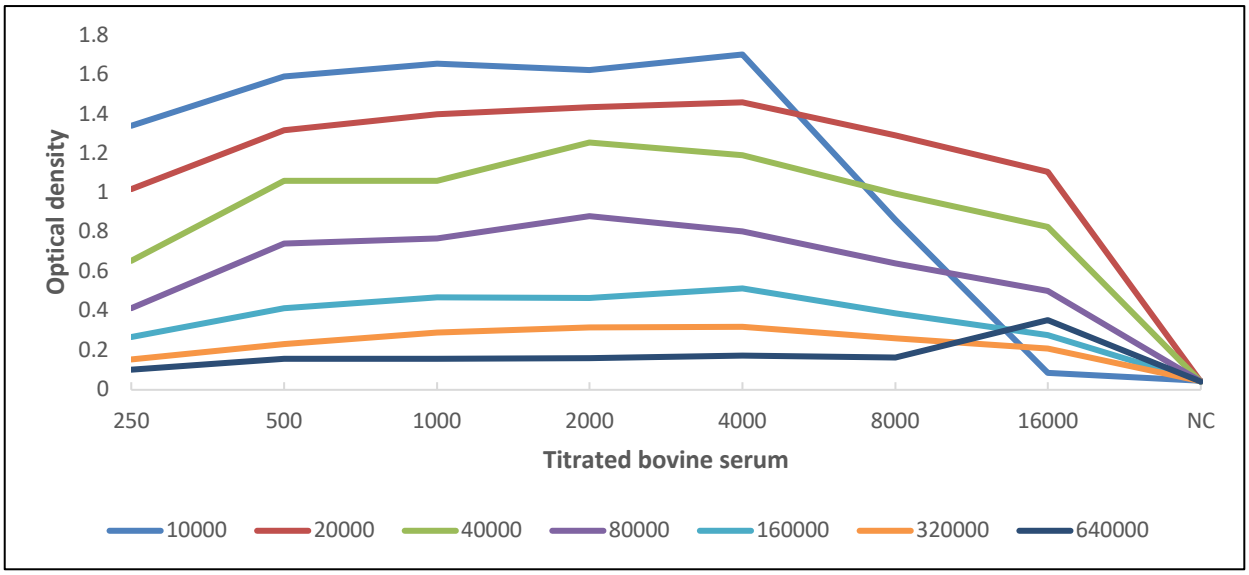

Figure 4-3: Results of the checker-board titration for bovine serum and protein A/G: HRP, plotting the conjugate dilutions. Bovine serum was titrated from 1:250 to 1:16000 and protein A/G: HRP from 1:10000 to 1:640000.

Maximum colour development was achieved by coating the plates with a serum dilution of up to 1:4000. To ensure saturation of antigen on the plates a serum coating dilution of 1:2000 was selected. The protein A/G: HRP dilution of 1:10000 maintained an OD of 1,6 at a 1:2000 serum dilution and was selected for further use in this project. At the same serum dilution the anti-bovine IgG: HRP dilution of 1: 2000 and 1:4000 for the anti-horse IgG: HRP were selected.

4.2.2 Titration to determine chaotrope concentration

To determine the optimum CT concentration that will result in less than 50% reduction of the colour reaction (OD), the CT was titrated and compared to wells that received PBS instead.

When comparing the effect of the different CT concentrations, it is evident that the CT concentrations ranging from 0,75 to 2 M dramatically disrupted the bonds between the bovine serum and both protein A/G: HRP and the anti-bovine IgG: HRP. The relative avidity

index (RAI), expressed as a percentage of the OD ratio, with and without the CT (Almanzar et al., 2013, Kneitz et al., 2004), is listed in Table 4-1 below.

Table 4-1: The relative avidity index (RAI) for each incremental chaotrope (CT) concentration, ranging from 2 M to 0,25 M NH_4SCN, was calculated for protein A/G: HRP at a dilution of 1:10000 and a 1:4000 dilution of the anti-bovine IgG: HRP on plates coated with 1:2000 diluted bovine serum. The RAI was expressed as a percentage of the CT-treated /PBS-treated OD values.

CT concentration (M)	RAI protein A/G: HRP	RAI anti-bovine IgG: HRP
2	11	20
1,75	11	34
1,5	14	22
1,25	20	21
1	27	23
0,75	45	29
0,5	62	37
0,25	88	55

At a 0,5 M CT concentration the average signal reduction was 38% resulting in a RAI of 62% for protein A/G: HRP and a reduction of 63% with a RAI of 37% for the anti-bovine IgG: HRP. Similarly, at 0,25 M CT concentration a reduction to 12% (average RAI was 88%) for protein A/G: HRP and 45% (average RAI was 55%) for the anti-bovine IgG: HRP was recorded. The 0,25 M CT concentration was used in the rest of the project.

4.3 Testing of wildlife serum for binding or cross-reactivity with recombinant protein A/G: HRP, rabbit anti-bovine IgG: HRP and goat anti-horse IgG: HRP

The OD values for the 42 ELISA plates are available in Annexure B, Section 4, arranged per species.

For three species, less than ten animals were tested, namely steenbok (n=3) and common duiker (n=2) due to limited available samples and only seven of the southern

reedbuck samples were tested. To ensure that inaccurate OD readings were not included in the results the raw data was examined and compared with notes on mistakes made in the laboratory. Mistakes, which included spillage and adding both treatments to certain wells, as in the case of gemsbok on the anti-horse IgG: HRP plate, were marked in red text in Annexure B and the values were removed from calculations. Seven species, namely Burchell's zebra, white rhinoceros, giraffe, impala, springbuck, common eland and gemsbok were affected by this, which resulted in either a single well per treatment or a reduction in sample numbers if both wells of a particular treatment were affected, for one or more of the conjugates. The CT treatment and PBS controls were swopped on the anti-horse IgG: HRP plate for nyala, when the first reagent was placed in the wrong rows (marked in blue), but due to timely corrective action the results were valid and the data could be included. Similarly, the control samples were added to a different location on the anti-bovine plate for tsessebe and roan, but corrective data management was implemented to ensure data integrity.

The frequency distribution (histogram) of the mean OD of the 27 wildlife species, as well as the controls, for the three conjugates are illustrated in Figure 4-4, Figure 4-5 and Figure 4-6 below, for protein A/G: HRP, the anti-bovine IgG: HRP and the anti-horse IgG: HRP, respectively.

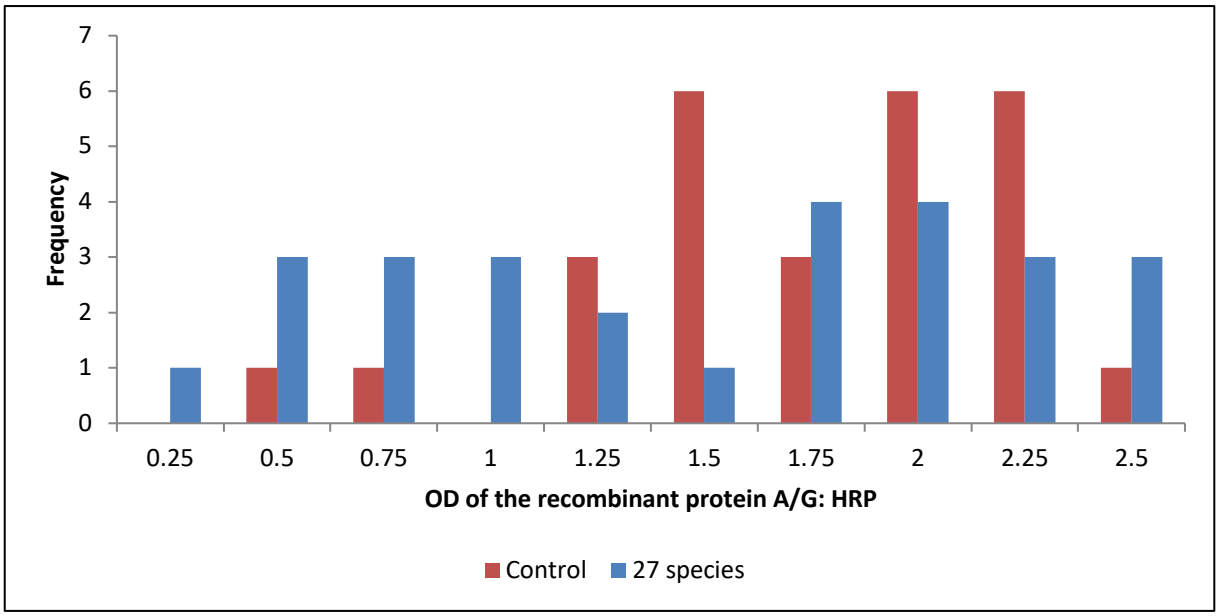

Figure 4-4: Frequency distribution of the mean OD for the bovine control and the mean OD for the 27 wildlife species for the recombinant protein A/G: HRP

The mean OD value for the 27 tested wildlife species tested for binding with protein A/G: HRP was 1,35±0,7 with a coefficient of variance of 0,52. The mean OD of the bovine control on the protein A/G: HRP plates was 1,62±0,7 and a very wide coefficient of variance of 0,5. The mean bovine control was situated below the 60th quantile of the distribution for the wildlife species tested for binding with protein A/G: HRP.

As illustrated in Figure 4-4 above, the protein A/G controls for two of the species (from the same ELISA plate) reacted well below the normal distribution curve namely species 2 Hartmann's zebra (OD: 0,41) and species 8 giraffe (OD: 0,63). The results for these two species may therefore be considered as invalid or suspicious.

When both species were excluded the mean control OD of the bovine control serum for the remaining 25 successfully tested species was 1,7±0,4 and a coefficient of variation of 0,23.

The mean OD value for the 27 wildlife species tested for cross-reactivity with the anti-bovine IgG: HRP was 0,28±0,17 with a coefficient of variance of 0,59. The mean OD of the bovine serum control on the anti-bovine IgG: HRP plates was 0,96±0,24 and a coefficient of variance of 0,25. The mean bovine control was situated far above the 100th quantile of the distribution for the wildlife species tested for cross-reactivity with the anti-bovine IgG: HRP.

The wider coefficient of variance for the bovine controls could once again be linked to lower bovine control values for the ELISA plate used to test Hartmann's mountain zebra (OD: 0,47) giraffe (OD: 0,51).

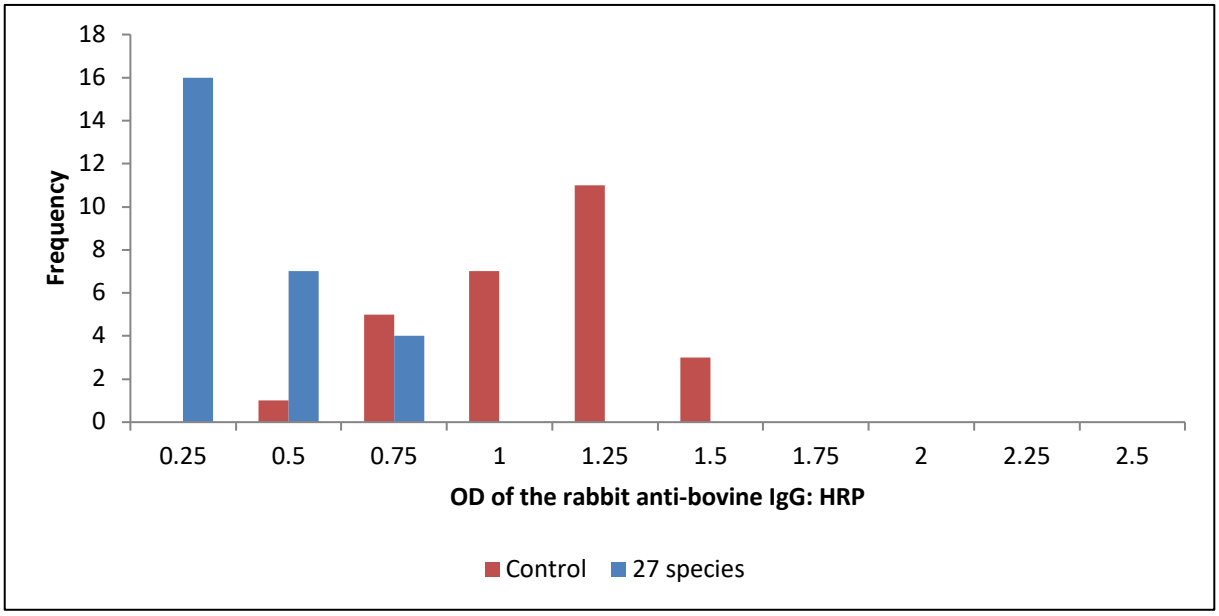

Figure 4-5: Frequency distribution of the mean OD for the bovine control and the mean OD for the 27 wildlife species for the rabbit anti-bovine IgG: HRP

The mean OD for the anti-horse IgG: HRP for the 27 successfully tested species was 0,61±0,69 and an extremely wide coefficient of variance of 1,15. As illustrated in Figure 4-6 below, good colour development was obtained in all the wells of the control equine serum, with a mean OD of 2,12±0,18 and a very narrow coefficient of variance of 0,08. The mean

control was located just above the 100th quantile for the anti-horse IgG: HRP and the results for all 27 species was considered valid for the anti-horse IgG: HRP.

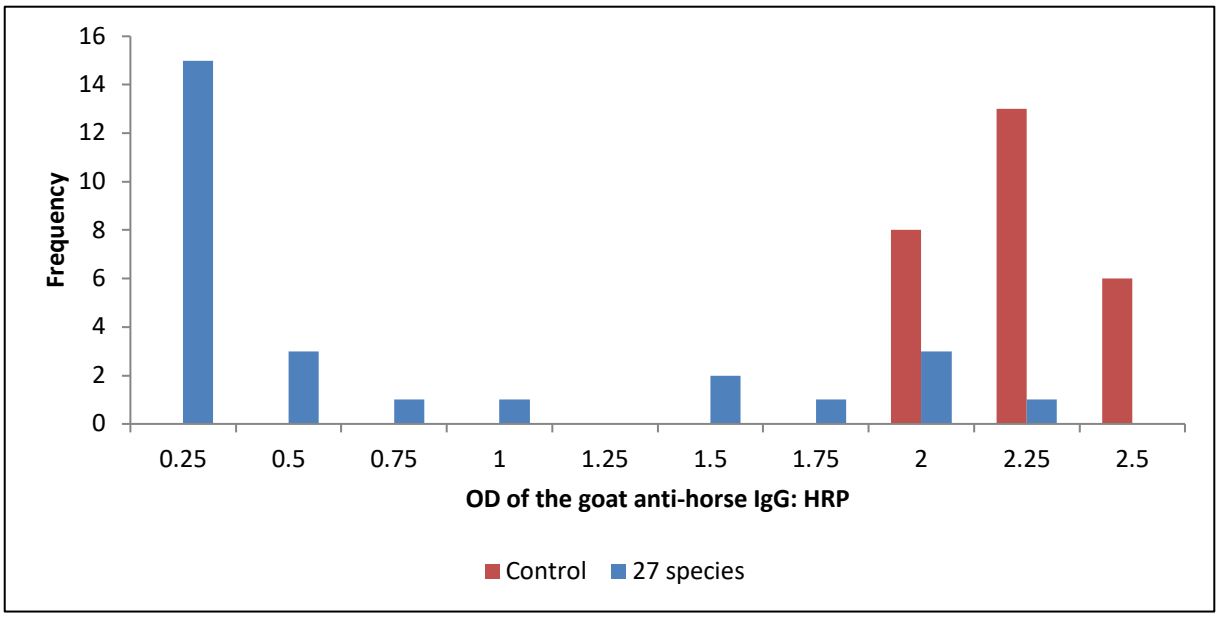

Figure 4-6: Frequency distribution of the mean OD for the equine control and the mean OD for the 27 wildlife species for the goat anti-horse IgG: HRP

The mean OD of the PBS-treated wells and the standard deviation (±SD) thereof for each species are reflected in Table 4-2, and Table 4-4 below for each of the conjugates. Variability in the SD was not only noted within (intra) and between (inter) species, but also between the different conjugates for the same species.

The normalised OD, which is the difference between the mean OD of each species (test samples) and the control on the same plate, was calculated for every conjugate, which resulted in a baseline value of zero for each of the respective bovine or equine controls, with positive and negative values for the normalised OD as an indication of either better or worse cross-reactivity compared to the control. These results, as well as the calculated t-value and p-values for the difference in mean between the test and control ODs, are also provided in Table 4-2, Table 4-3 and Table 4-4 below for each conjugate.

Table 4-2: Protein A/G: HRP statistics: The number of samples the calculations are based on, the mean OD values of the PBS-treated wells of each wildlife species ± the SD, the OD of the PBS-treated bovine control serum for that species, followed by the normalised OD, calculated by subtracting the mean control OD from the mean OD for each species. This resulted in a normalised baseline value of zero for the control with positive values for species binding better with protein A/G and negative values for those binding poorer than the bovine control. A two sided t-test was performed to compare the means of each species and their related controls, of which the t-value and p-value test statistics are included. * Invalid bovine control on these plates resulted in data that cannot be validated.

	Specie	**Sample size (n)**	**Mean OD**	***±SD***	**Bovine control**	**Normalised OD**	**t-value**	**p-value**
1	African elephant	10	1,954	0.295	2,066	-0,112	-1.4247	0.171
2	Hartmann's zebra	10	0,702	0.171	*0,413	0,289	4.543	0.000682
3	Burchell's zebra	10	1,192	0.114	1,093	0,100	2.1358	0.05674
4	White rhinoceros	10	2,395	0.041	2,207	0,188	4.4647	0.01359
5	Black rhinoceros	10	1,524	0.168	1,092	0,432	7.142	1.2E-05
6	Common warthog	10	2,415	0.059	2,172	0,243	8.4857	3.44E-05
7	Hippopotamus	10	2,023	0.153	2,082	-0,059	-0.8452	0.4205
8	Giraffe	10	0,391	0.163	*0,629	-0,237	-3.8695	0.002274
9	Impala	10	0,287	0.155	1,645	-1,358	-25.004	1.61E-11
10	Hartebeest	10	0,863	0.146	1,272	-0,409	-7.088	1.8E-05
11	Black wildebeest	10	0,748	0.350	1,281	-0,533	-3.814	0.002828
12	Blue wildebeest	10	1,647	0.212	1,989	-0,342	-3.9485	0.00241
13	Tsessebe	10	0,871	0.155	1,080	-0,209	-2.5019	0.0439
14	Blesbok	10	1,697	0.524	1,910	-0,213	-1.1496	0.273
15	Springbok	10	1,621	0.428	1,912	-0,291	-1.9509	0.07548
16	Steenbok	3	2,067	0.226	1,681	0,386	2.7035	0.07895
17	African buffalo	10	2,306	0.220	2,230	0,077	1.0286	0.3254
18	Nyala	10	0,851	0.403	1,261	-0,410	-2.7954	0.01618
19	Common eland	10	1,040	0.400	1,711	-0,671	-5.0701	0.000416
20	Bushbuck	10	1,866	0.246	2,305	-0,439	-5.2137	0.000257
21	Greater kudu	10	0,053	0.025	1,931	-1,877	-31.013	5.69E-05
22	Common duiker	2	1,999	0.047	1,467	0,532	9.2687	0.000883
23	Roan antelope	10	0,486	0.152	1,289	-0,804	-10.503	1.34E-05
24	Sable antelope	10	2,001	0.362	2,007	-0,006	-0.0632	0.9508
25	Gemsbok	9	1,252	0.321	1,785	-0,533	-4.8852	0.000995
26	Waterbuck	10	0,555	0.213	1,392	-0,836	-8.0747	5.24E-05
27	Southern reedbuck	7	1,775	0.904	1,840	-0,065	3.9702	0.8583

Table 4-3: Anti-bovine IgG: HRP statistics: The number of samples the calculations are based on, the mean OD values of the PBS-treated wells of each wildlife species ± the SD, the OD of the PBS-treated bovine control serum for that species, followed by the normalised OD, calculated by subtracting the mean control OD from the mean OD for each species. This resulted in a normalised baseline value of zero for the control with positive values for species with better cross-reaction with anti-bovine IgG: HRP and negative values for those with poorer cross-reactivity than the bovine control. A two sided t-test was performed to compare the means of each species and their related controls, of which the t-value and p-value test statistics are included. * Invalid bovine control on these plates resulted in data that cannot be validated.

	Specie	**Sample size (n)**	**Mean OD**	***±SD***	**Bovine control**	**Normalised OD**	**t-value**	**p-value**
1	African elephant	10	0,045	*0.003*	1,115	-1,070	-18.36	0.000352
2	Hartmann's zebra	10	0,107	*0.032*	*0,475	-0,368	-10.672	0.000772
3	Burchell's zebra	10	0,137	*0.036*	0,648	-0,511	-27.038	4.55E-08
4	White rhinoceros	9	0,080	*0.033*	0,934	-0,854	-27.906	1.22E-05
5	Black rhinoceros	10	0,129	*0.015*	0,683	-0,553	-16.545	0.000382
6	Common warthog	10	0,086	*0.019*	0,973	-0,887	-72.57	7.33E-09
7	Hippopotamus	10	0,231	*0.043*	1,348	-1,117	-18.327	0.000188
8	Giraffe	10	0,196	*0.036*	*0,513	-0,317	-15.94	3.25E-06
9	Impala	9	0,204	*0.056*	1,112	-0,908	-19.903	2.9E-05
10	Hartebeest	10	0,246	*0.060*	0,858	-0,613	-20.906	6.05E-08
11	Black wildebeest	10	0,228	*0.045*	0,668	-0,440	-14.798	2.73E-05
12	Blue wildebeest	10	0,391	*0.164*	1,091	-0,701	-10.439	6.23E-07
13	Tsessebe	10	0,257	*0.045*	0,888	-0,632	-32.357	1.587e-10
14	Blesbok	10	0,323	*0.168*	1,091	-0,768	-13.491	2.91E-08
15	Springbok	7	0,384	*0.224*	0,970	-0,586	-8.005	1.1E-05
16	Steenbok	3	0,692	*0.265*	1,260	-0,567	-2.458	0.05771
17	African buffalo	9	0,513	*0.144*	1,037	-0,524	-8.8678	2.83E-06
18	Nyala	10	0,220	*0.071*	0,770	-0,550	-17.485	2.06E-08
19	Common eland	7	0,162	*0.033*	1,105	-0,943	-27.079	1.32E-05
20	Bushbuck	10	0,195	*0.053*	1,093	-0,898	-15.668	0.0002
21	Greater kudu	10	0,375	*0.106*	1,207	-0,832	-15.526	1E-06
22	Common duiker	2	0,524	*0.043*	1,089	-0,565	-12.313	0.000667
23	Roan antelope	10	0,200	*0.044*	0,839	-0,638	-10.503	0,000013
24	Sable antelope	10	0,558	*0.056*	1,307	-0,748	-20.967	6.83E-10
25	Gemsbok	9	0,477	*0.235*	1,170	-0,693	-8.7096	2.73E-06
26	Waterbuck	10	0,218	*0.035*	0,677	-0,460	-23.39	4.39E-07
27	Southern reedbuck	7	0,499	*0.259*	1,096	-0,597	-6.0725	0.00086

* Invalid bovine control on these plates resulted in data that cannot be validated.

Table 4-4: Anti-horse IgG: HRP statistics: The number of samples the calculations are based on, the mean OD values of the PBS-treated wells of each wildlife species ± the SD, the OD of the PBS-treated equine control serum for that species, followed by the normalised OD, calculated by subtracting the mean control OD from the mean OD for each species. This resulted in a normalised baseline value of zero for the control with positive values for species with better cross-reaction with the anti-horse IgG: HRP and negative values for those with poorer cross-reactivity than the equine control. A two sided t-test was performed to compare the means of each species and their related controls, of which the t-value and p-value test statistics are included. * Invalid bovine control on these plates resulted in data that cannot be validated.

	Specie	**Sample size (n)**	**Mean OD**	***±SD***	**Equine control**	**Normalised OD**	**t-value**	**p-value**
1	African elephant	10	1,306	*0.094*	2,079	-0,773	-26.268	1.62E-12
2	Hartmann's zebra	10	1,815	*0.140*	1,918	-0,104	-1.7608	0.1084
3	Burchell's zebra	10	1,939	*0.131*	1,844	0,095	1.673	0.1268
4	White rhinoceros	10	2,031	*0.050*	2,154	-0,123	-2.398	0.08042
5	Black rhinoceros	10	1,897	*0.064*	1,936	-0,039	-0.70208	0.5215
6	Common warthog	10	1,581	*0.132*	2,294	-0,712	-10.952	7.47E-06
7	Hippopotamus	10	1,442	*0.099*	2,037	-0,596	-6.2233	0.004242
8	Giraffe	10	0,067	*0.008*	1,908	-1,841	-52.199	0.000014
9	Impala	10	0,225	*0.119*	2,227	-2,002	-13.074	0.000506
10	Hartebeest	10	0,101	*0.039*	2,009	-1,908	-91.484	3.12E-11
11	Black wildebeest	10	0,289	*0.179*	1,895	-1,606	-23.305	4.01E-11
12	Blue wildebeest	10	0,100	*0.034*	2,370	-2,270	-21.193	0.000201
13	Tsessebe	10	0,145	*0.103*	1,922	-1,777	-47.034	5.58E-15
14	Blesbok	10	0,121	*0.023*	2,438	-2,317	-13.095	0.000946
15	Springbok	7	0,221	*0.210*	2,389	-2,169	-29.289	2.24E-12
16	Steenbok	3	0,228	*0.197*	2,131	-1,902	-15.965	0.001755
17	African buffalo	10	0,073	*0.030*	2,237	-2,164	-66.247	1.16E-06
18	Nyala	10	0,778	*0.325*	2,010	-1,232	-11.945	6.79e-07
19	Common eland	10	0,398	*0.224*	2,223	-1,825	-18.543	1.1E-08
20	Bushbuck	10	0,243	*0.092*	2,184	-1,941	-17.809	0.000155
21	Greater kudu	10	0,650	*0.294*	2,059	-1,409	-12.886	2.28E-08
22	Common duiker	2	0,139	*0.052*	2,129	-1,990	-50.707	0.004872
23	Roan antelope	10	0,100	*0.020*	1,951	-1,852	-79.763	8.66E-07
24	Sable antelope	10	0,068	*0.070*	2,143	-2,075	-45.835	5.54E-06
25	Gemsbok	9	0,094	*0.035*	2,281	-2,187	-29.191	0.01863
26	Waterbuck	10	0,131	*0.034*	1,957	-1,826	-47.386	4.49E-06
27	Southern reedbuck	7	0,252	*0.243*	2,419	-2,167	-22.502	6.92E-08

Highly significant p-values ($p<0,05$) confirmed a difference in cross-reactivity from the respective control, binding with the conjugate either better (positive t-value) or worse

(negative t-value) than the control. For p>0,05 there was not enough evidence to reject the null hypothesis and the binding capacity was found comparable to the respective control species.

The calculated normalised OD of the different species are illustrated in Figure 4-7, Figure 4-8 and Figure 4-9 below for each of the three conjugates, with the OD on the y-axis and the value of the control serum equal to zero. The error bars illustrates the 95% confidence intervals calculated by the two-sided t-test and indicating the significance of the differences between the test and control values if not spanning zero.

In Figure 4-7 below it is evident that six species, including both zebra species, both rhinoceros species, warthog and duiker (n=2) performed better than the control bovine serum with higher OD readings than the control and significant p-values. The difference between the control serum and eight species including elephant, hippopotamus, blesbok, springbok, steenbok (n=3) and buffalo, sable and reedbuck was not significant (not enough evidence to reject the null hypothesis).

Please be reminded that invalid bovine control on one of these plates resulted in data for the Hartmann's mountain zebra and giraffe not validated. These are indicated with a star on the graphs below.

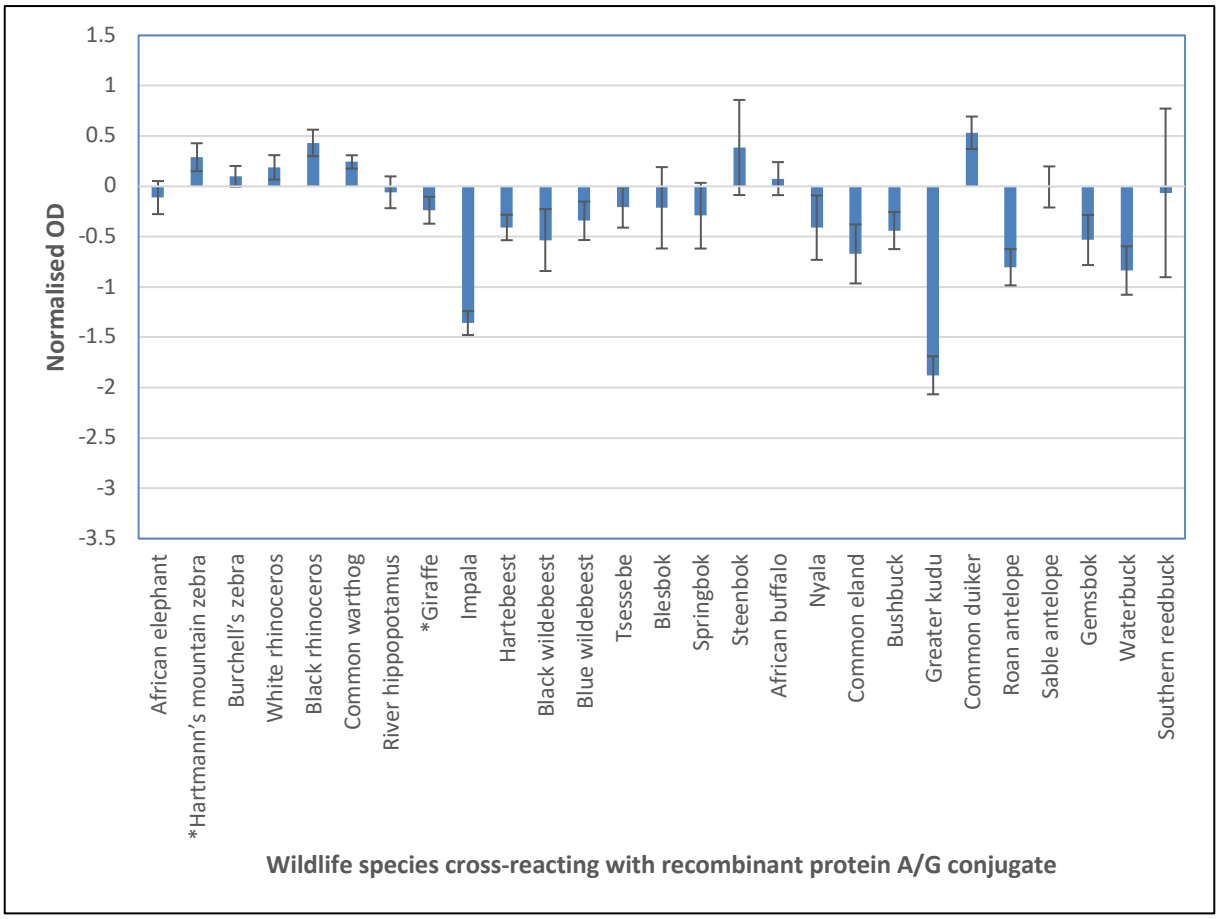

Figure 4-7: Differences in binding of recombinant protein A/G: HRP with serum IgG of various wildlife species compared to bovine serum IgG. The differences were normalised by subtracting the mean OD obtained for the bovine serum from the mean OD for each tested species (n=10 for most species). The zero line represents the bovine control. Positive values indicate higher binding capacity than the bovine serum while negative values indicate less binding. The error bars represent the 95% confidence intervals. All differences between the means were highly significant ($p<0,05$) except where the error bars spanned zero, confirming that the differences were not significant ($p\geq0,05$). * Invalid bovine control on these plates resulted in data that cannot be validated.

In Figure 4-8 below it is evident that all the species reacted significantly less ($p<0,05$) to the anti-bovine IgG: HRP than the control bovine serum. The difference between the control bovine serum and species 16 (steenbok n=3) was not significant (not enough evidence to reject the null hypothesis).

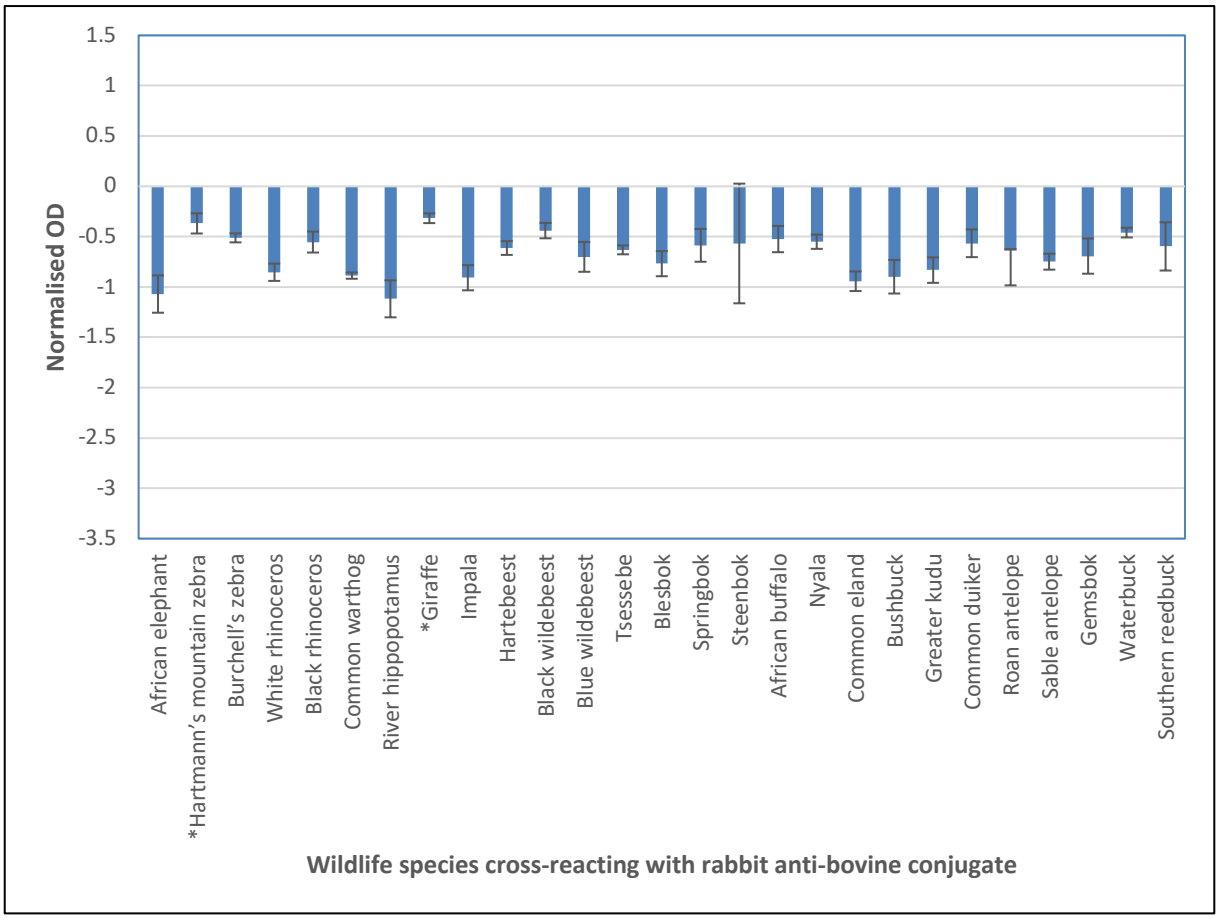

Figure 4-8: Differences in cross-reactivity of various wildlife species serum IgG and the rabbit anti-bovine IgG: HRP as compared to bovine serum IgG. The differences were normalised by subtracting the mean OD obtained for the bovine serum from the mean OD for each tested species (n=10 for most species). The zero line represents the bovine control. Positive values indicate higher binding capacity than the bovine serum while negative values indicate less binding. The error bars represent the 95% confidence intervals. All differences between the means were highly significant ($p<0.05$) except where the error bars included zero, confirming that the differences were not significant ($p\geq0.05$). * Invalid bovine control on these plates resulted in data that cannot be validated.

From Figure 4-9 below, illustrating cross-reactivity to the anti-horse IgG: HRP, it is interesting to note that only the Burchell's zebra performed slightly better than the control horse serum. The difference between the control equine serum and Hartman's mountain zebra, Burchell's zebra, white rhino and black rhino was not significant ($p>0,05$) and therefore not enough evidence to reject the null hypothesis and their cross-reactivity with this conjugate were found to be comparable with the equine control.

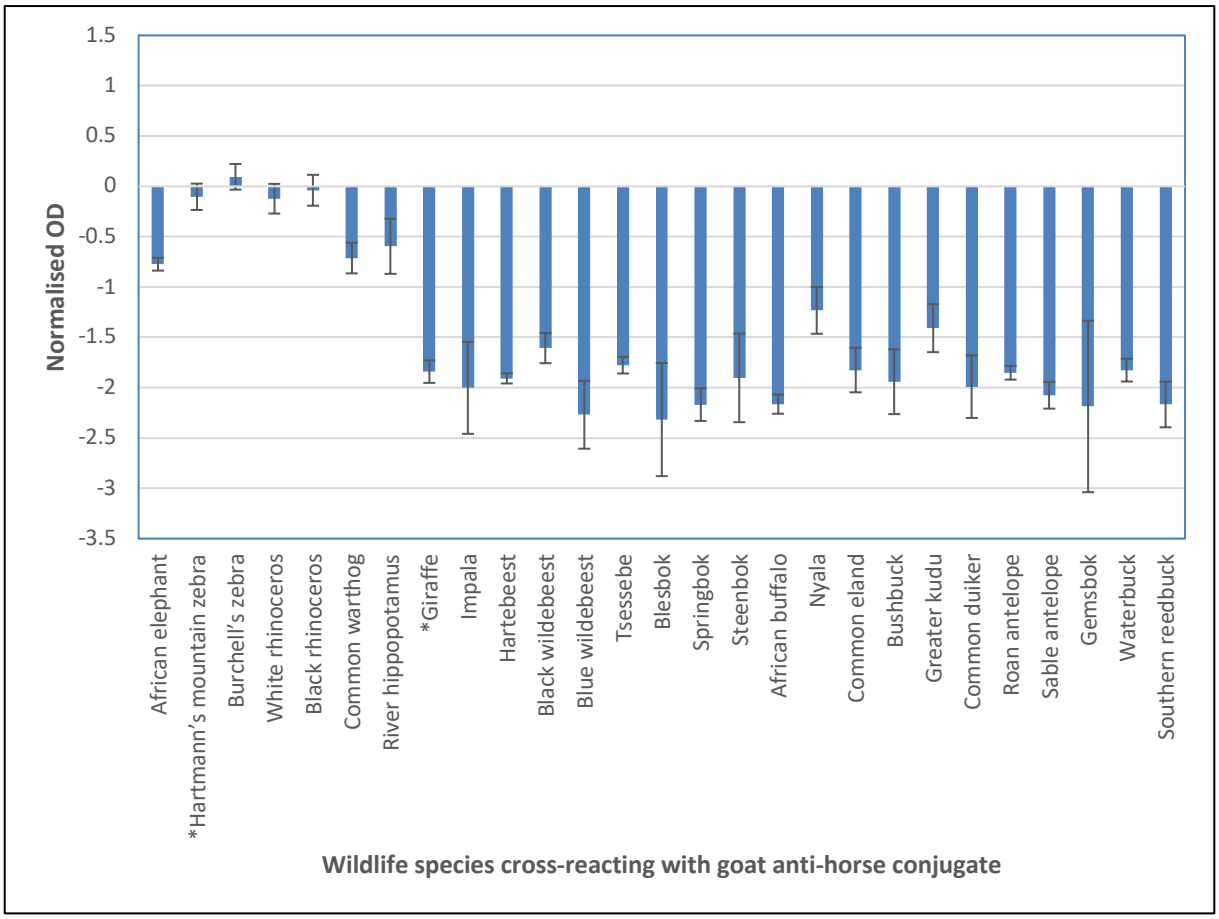

Figure 4-9: Differences in cross-reactivity of various wildlife species serum IgG and the goat anti-horse IgG: HRP as comparted to equine serum IgG. The differences were normalised by subtracting the mean OD obtained for the equine serum from the mean OD for each tested species (n=10 for most species). The zero line represents the equine control. Positive values indicate higher binding capacity than the equine serum while negative values indicate less binding. The error bars represent the 95% confidence intervals All differences between the means were highly significant ($p<0.05$) except where the error bars included zero, confirming that the differences were not significant ($p\geq0.05$).

Despite the samples size of duiker (species 22) being extremely small (n=2) the differences with the positive controls were highly significant for all three conjugates.

These results, however, do not give any indication of the functional avidity between the different species and the respective conjugates. The relative avidity index (RIA) was subsequently calculated as the ratio of the mean OD values from the NH_4SCN treaded wells to that of the PBS-treated wells. The RAI for each species and the respective control serum

and conjugate are indicated in Figure 4-10, Figure 4-11 and Figure 4-12. RAI was not calculated for species that demonstrated poor cross-reactivity (OD <50% of control value).

The average RAI for the bovine control samples over all 14 protein A/G: HRP plates was 0,51±0,13. In Figure 4-10 below it is clear that, at the NH_4SCN concentration of 0,25 M, thirteen species demonstrated a higher functional avidity for protein A/G: HRP compared to the control bovine serum, with white rhinoceros and warthog above 0,8.

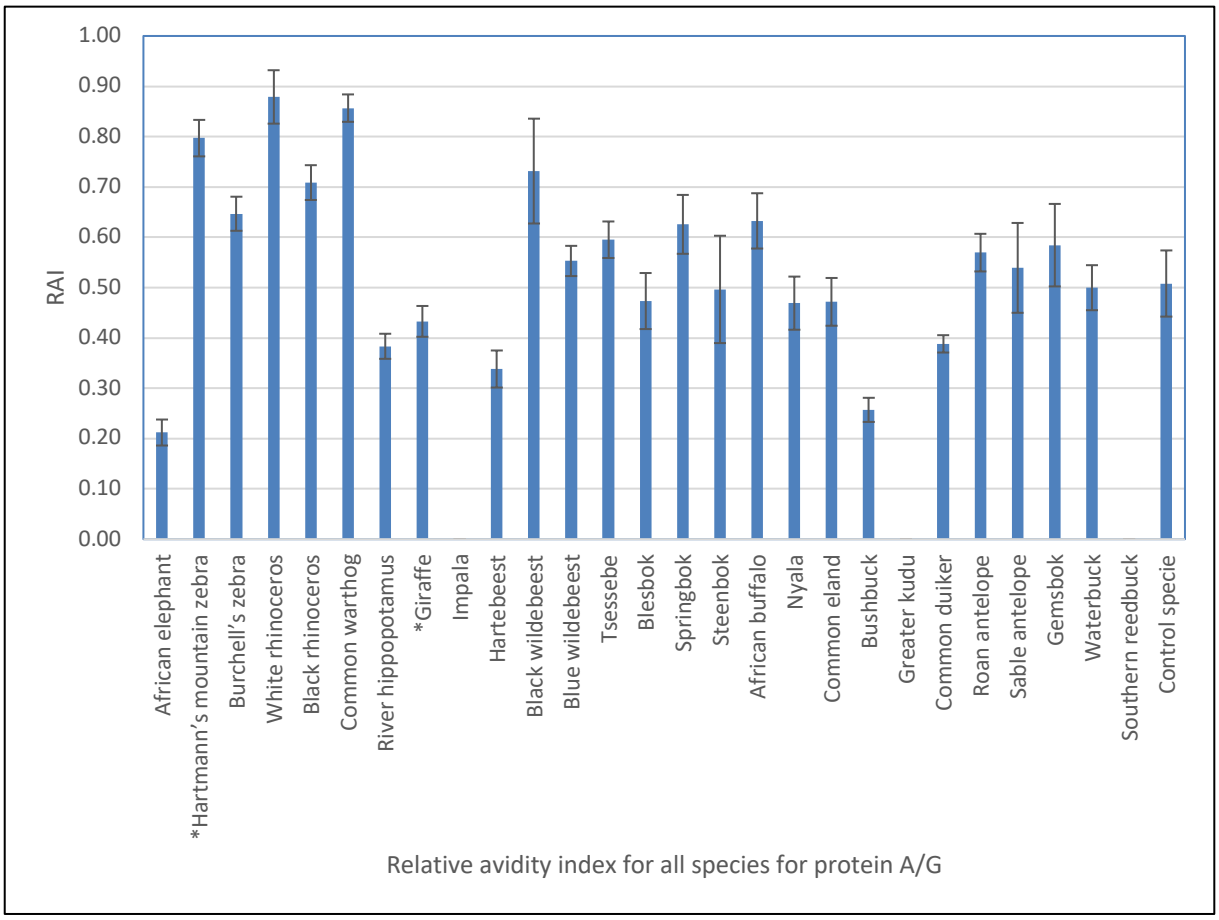

Figure 4-10: Differences in the relative avidity index (RAI) of various wildlife species (n=10) and the bovine control serum for recombinant protein A/G: HRP. For each animal duplicate wells were treated with a final concentration of 0,25 M NH_4SCN and duplicate wells received PBS buffer instead. The RAI was calculated from the ratio of the mean OD values from the NH_4SCN-treated wells to that of the PBS-treated wells. No RAI was calculated for species that showed poor reactivity to the protein A/G: HRP and these are indicated with a zero value. The error bars represent the standard deviation.
* Invalid bovine control on these plates resulted in data that cannot be validated.

The average RAI for the bovine control samples over the 14 anti-bovine IgG: HRP plates was 0,37±0,08 . In Figure 4-11 below it is evident that, of the three species that cross-reacted more than 48% with the rabbit anti-bovine IgG: HRP as compared to the bovine control serum, buffalo demonstrated the best RAI (0,25), which is 67,59% of the mean RAI of the bovine control serum (0,37).

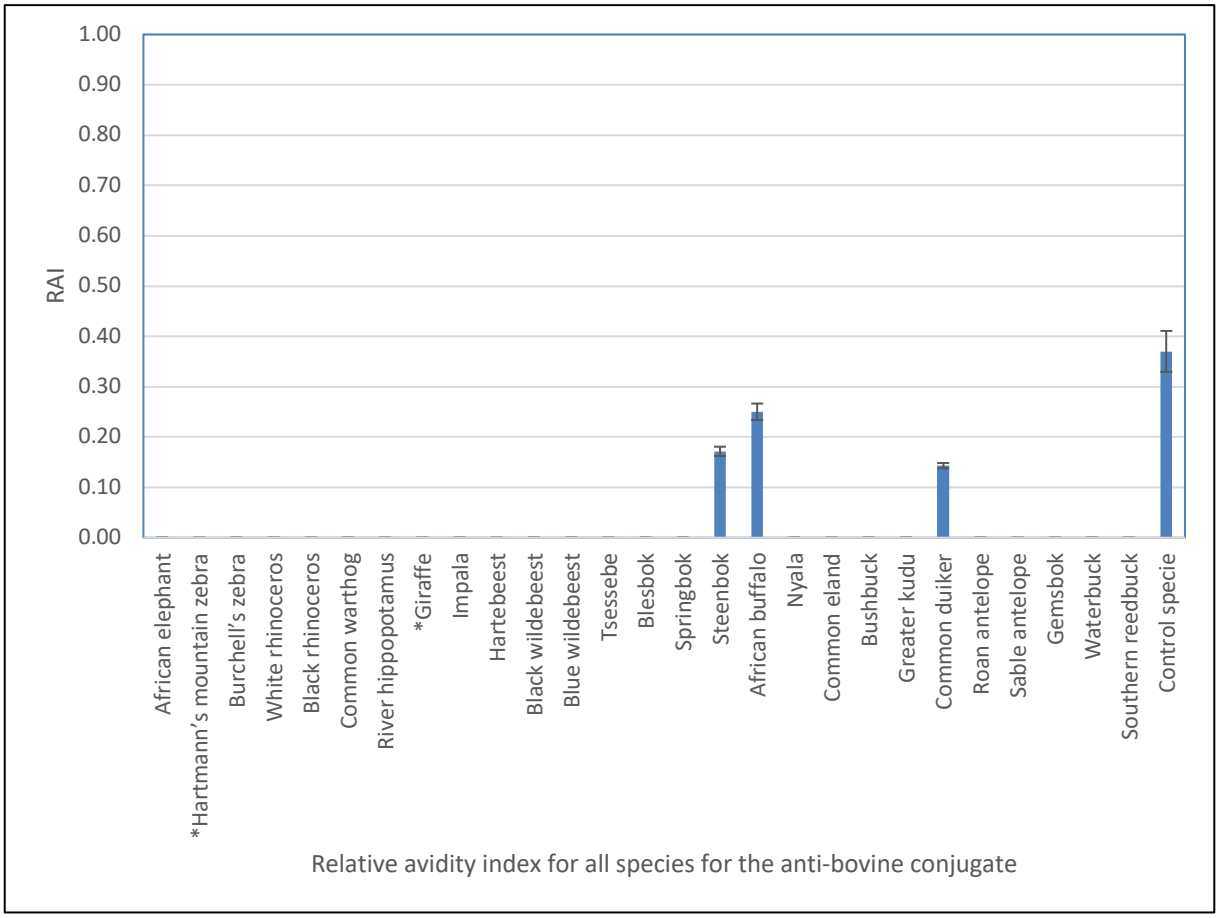

Figure 4-11: Differences in the relative avidity index (RAI) of various wildlife species (n=10) and the bovine control serum for the rabbit anti-bovine IgG: HRP. For each animal duplicate wells were treated with a final concentration of 0.25 M NH_4SCN and duplicate wells received PBS buffer instead. The RAI was calculated from the ratio of the mean OD values from the NH_4SCN-treated wells to that of the PBS-treated wells. No RAI was calculated for species that showed poor reactivity to the rabbit anti-bovine IgG: HRP and these are indicated with a zero value. The error bars represent the standard deviation. * Invalid bovine control on these plates resulted in data that cannot be validated.

The average RAI for the equine control samples over all 14 anti-horse IgG: HRP plates was 0,34±0,18. As illustrated in Figure 4-12 below, of the six species that cross-reacted

more than 50% with the anti-horse IgG: HRP compared to the equine control serum, three demonstrated a better RAI than the equine control (0,34), including both zebra species, with Hartmann's mountain zebra (0,54), Burchell's zebra (0,5) and black rhino (0,41).

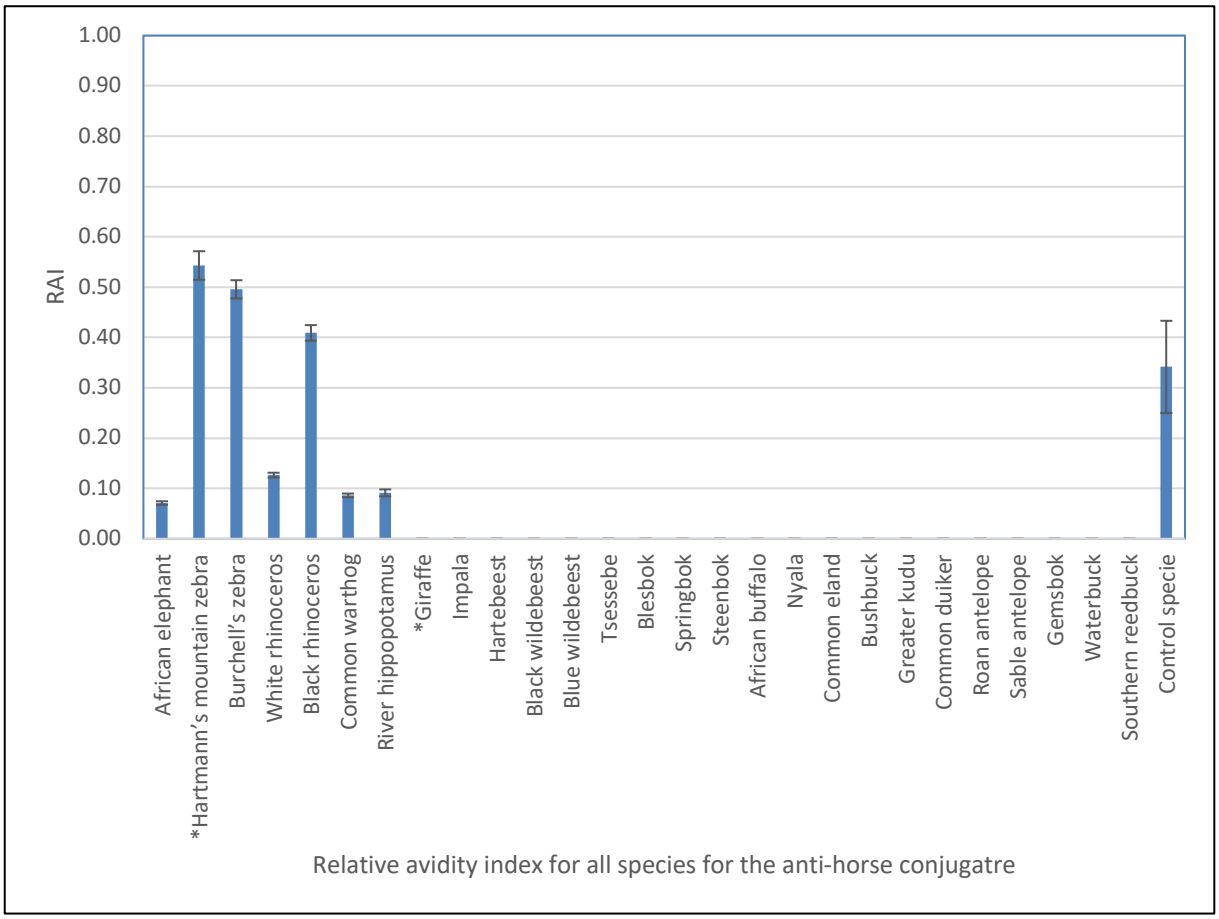

Figure 4-12: Differences in the relative avidity index (RAI) of various wildlife species (n=10) and the equine control serum for the goat anti-horse IgG: HRP. For each animal duplicate wells were treated with a final concentration of 0,25 M NH_4SCN and duplicate wells received PBS buffer instead. The RAI was calculated from the ratio of the mean OD values from the NH_4SCN-treated wells to that of the PBS-treated wells. No RAI was calculated for species that showed poor reactivity to the goat anti-horse IgG: HRP and these are indicated with a zero value. The error bars represent the standard deviation.

In an attempt to quantify the usefulness of each conjugate, several strategies were examined, such as normalising the data by calculating the sample to positive ratio (SP) for the individual test sera, which was then added to the RAI to define an index (usefulness index) to rate conjugates RAI to define an index (usefulness index) to rate conjugates, as demonstrated as a radar chart in Figure 4-13 below. For each of the three conjugates the 27 wildlife species are indexed respectively to the control, with lower values in the centre

and higher ratings at the rim of the radar chart. In this summation index the differences between the conjugates are clearly accentuated.

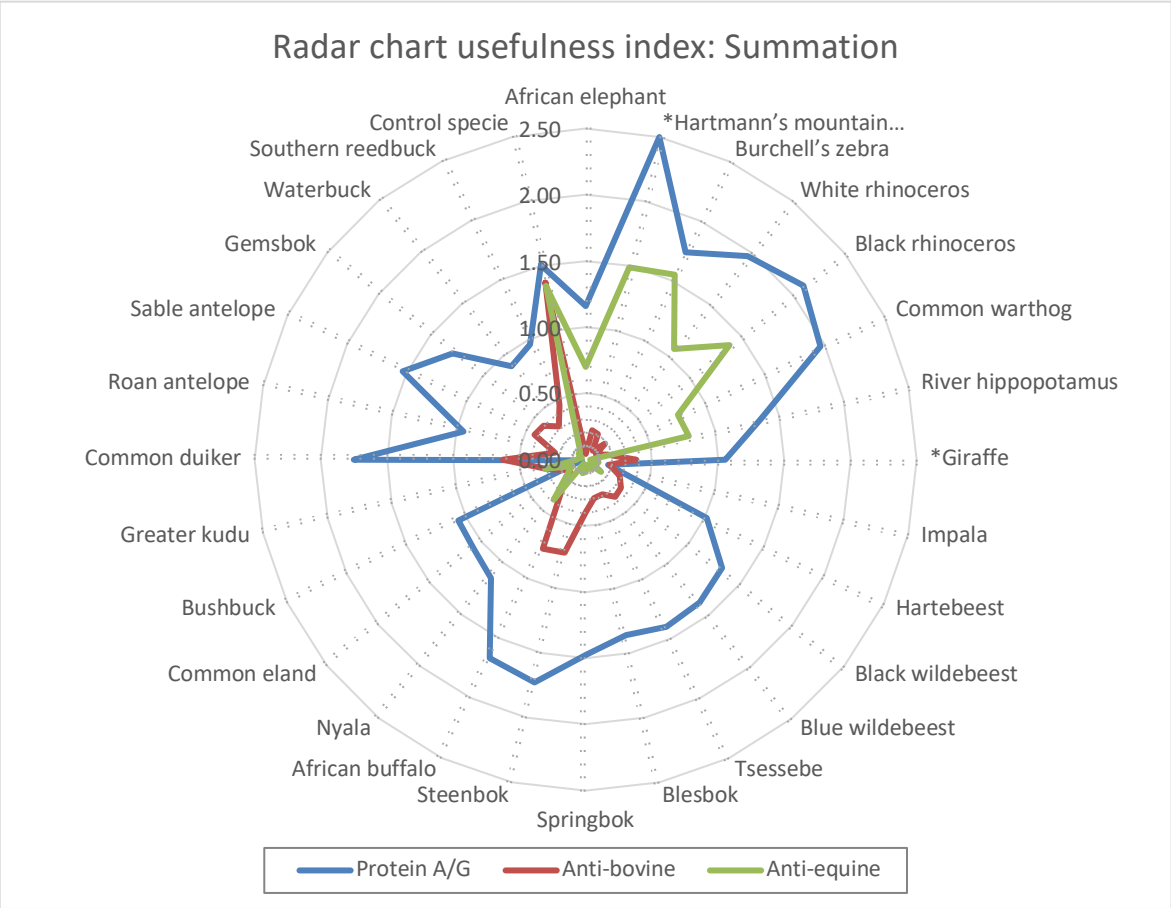

Figure 4-13: Radar chart of usefulness index for the three conjugates using the sum of the sample to positive ratio (SP) of the ODs and the RAI to rate the conjugates. * Invalid bovine controls on the recombinant protein A/G: HRP and rabbit anti-bovine IgG: HRP plates resulted in data that cannot be validated.

4.4 References

ALMANZAR, G., OTTENSMEIER, B., LIESE, J. & PRELOG, M. 2013. Assessment of IgG avidity against pertussis toxin and filamentous hemagglutinin via an adapted enzyme-linked immunosorbent assay (ELISA) using ammonium thiocyanate. *Journal of Immunological Methods,* 387**,** 36-42.

DAUNER, J. G., PAN, Y., HILDESHEIM, A., KEMP, T. J., PORRAS, C. & PINTO, L. A. 2012. Development and application of a GuHCl-modified ELISA to measure the avidity of anti-HPV L1 VLP antibodies in vaccinated individuals. *Molecular and Cellular Probes,* 26**,** 73-80.

KNEITZ, R.-H., SCHUBERT, J., TOLLMANN, F., ZENS, W., HEDMAN, K. & WEISSBRICH, B. 2004. A new method for determination of varicella-zoster virus immunoglobulin G avidity in serum and cerebrospinal fluid. *BMC infectious diseases,* 4**,** 33.

Chapter 5 Discussion

The purpose of this study was to evaluate the cross reactivity of commercially available polyvalent anti-bovine IgG: HRP, anti-horse IgG: HRP and binding of recombinant protein A/G: HRP with the serum IgG of various African wildlife species. Serum from 27 herbivore and hoof stock wildlife species were obtained and a direct ELISA was performed on 10 animals from each species for each of the three selected conjugates. Responses in wildlife to anti-bovine IgG: HRP and recombinant protein A/G: HRP were respectively expressed relative to the response to bovine serum and responses to the anti-horse IgG: HRP were expressed relative to equine serum. A relative avidity index as well as a usefulness index was calculated for each of the conjugates and each of the wildlife species.

Thirteen wildlife species performed better than or equal to the bovine control with recombinant protein A/G: HRP while the rest of the species performed significantly lower than the bovine control. In contrast, the rabbit anti-bovine IgG: HRP was found not to be useful for any of the wildlife species tested in this study. The goat anti-horse IgG: HRP was found to be equivalent to the equine control in four species, however the recombinant protein A/G: HRP performed better in all four these species.

These findings were based on a combination of the measured cross-reactivity (OD) in relation to a known homologues control serum (normalised OD) and the functional avidity, which is a measure of the binding affinity expressed as the relative avidity index (RAI). Ammonium thiocyanate (NH_4SCN) was used as a bond-disrupting or chaotropic agent to calculate the RAI. The summation of the OD and the RAI, both as a ratio of the homologous control was indexed and used to compare and rate the three conjugates evaluated in this

study. In the absence of specific anti-species conjugates for wildlife species, the quantification of the usefulness of alternative commercially available enzyme conjugates may assist in the development of primary binding assays that was previously not possible. The findings also contribute to the current known scope of the binding affinity of recombinant protein A/G: HRP as published in datasheets for antibody-binding proteins (ThermoScientific, 2016). A standard conjugate evaluation process is therefore proposed to assist future evaluation of commercial conjugates for wildlife species.

Ammonium thiocyanate (NH_4SCN) was confirmed as an appropriate chaotrope (CT) for use in this study. The effect of NH_4SCN on the reactivity of HRP enzyme was first evaluated. This was done by coating the HRP enzyme conjugate to an ELISA plate and then incubating it with different concentrations of the chaotrope. The chaotrope was then removed by washing before the substrate was added. This probably resulted in spontaneous recovery of any conformational changes or reactivity in the enzyme as there was no difference in the colour development when compared to wells that were incubated with PBS instead (Figure 4-2). However, the combined effect of the carbonate buffer (CB) as coating diluent and the higher CT concentrations (2,0 M and 1,75 M) probably resulted in more harsh conditions that led to irreversible conformational changes in the enzyme that were directly proportional to the colour development (Figure 4-1).

This experiment demonstrated that the HRP enzyme remained stable when subjected to various concentrations of the chaotrope and that there was no significant difference in colour development when the HRP enzyme was incubated with either the chaotrope or the PBS. Therefore any reduction in colour development when a specific anti-species conjugate binds with antibody in the presence of a predetermined chaotrope concentration should

be due to reduced binding and not an inhibitory effect on the enzyme. The ratio between colour development in the presence of chaotrope to colour development without chaotrope could be used as a relative avidity index to indicate binding strength between the enzyme conjugate and antibody from the target species.

Dauner et al. (2012) reported using NH_4SCN concentrations from 0,25 to 1,75 M and Almanzar et al. (2013) reported that the difference between species was most evident between 1,5 and 2 M concentration.

Dauner et al. (2012) reported that serum dilution did not seem to effect the RAI and similarly Almanzar et al. (2013) concluded that the initial Ab concentration did not influence the outcome of this index, unless the Ab concentration was extremely low. The chosen serum dilution of 1:2000 for this study was comparable with similar published studies (Kelly et al., 1993, Stöbel et al., 2002). In contrast, however, CT concentration as well as the time and temperature of incubation were reported to have a considerable impact on the stability of Ag-Ab bonds (Almanzar et al., 2013) and the CT concentration, temperature and time of incubation were therefore standardised to induce uniform CT effect. In this study the Ab concentration was not measured, but assumed to be relatively constant due to the constant serum dilution factor. This assumption may, however, warrant consideration in validation studies. It is also important to ensure that background staining is not allowed to artificially influence RAI calculations when low cross-reactivity is encountered.

PBS (Ferreira and Katzin, 1995) and PBST (Dauner et al., 2012) were both previously described as diluents for NH_4SCN. In this study, however, distilled water was used. It may be prudent to compare the effect of PBS, PBST and distilled water as NH_4SCN diluents,

taking special note of the effect of changes in pH and molarity that may influence the chaotropic effect of NH_4SCN. A standard recommended diluent for similar future research projects will assist in comparative assessment of results.

As specific target species, bovine serum was the obvious choice of control for the rabbit anti-bovine IgG: HRP, as was equine serum for the goat anti-horse IgG: HRP. For this study bovine serum was also used as control for the recombinant protein A/G: HRP. This choice was based on the manufacturers datasheet rating as “strong binding” (ThermoScientific, 2016), as well as a previous publication (Kramsky et al., 2003a) and on availability. The control serums were not representative of the bovine and equine species, as they only represented limited samples that had been pooled, but it was a constant homogenous sample used across all tested species for a specific conjugate, to ensure comparability between test species for each conjugate. The approximate usefulness index value of 1,5 reached by all three controls was the basis on which the conjugates were compared.

Two wildlife species, namely Hartmann’s mountain zebra and giraffe, were tested on the same plates. The mean OD of the bovine control on the protein A/G: HRP plate (0,41 and 0,63 respectively) and the anti-bovine IgG: HRP plate (0,47 and 0,51 respectively) were suspiciously low for both these plates. This is evident in the frequency distribution of the control ODs in Figure 4-4 and Figure 4-5 and may have been the result of an unnoticed laboratory error. The most probable explanation, assuming these plates were the first to be coated during a particular laboratory session, is that the homologues control bovine serum sample was initially not sufficiently agitated and resulted in coating with a lower IgG concentration of the control wells. This laboratory error was not detected by the

inexperienced student at the time. Re-testing was indicated but not possible within the limited study period. Data for the protein A/G: HRP and the anti-bovine IgG: HRP for these two species were therefore not considered valid. The equine control on the anti-horse IgG: HRP plate for these two species, which was prepared in the same session, was, however, within the expected OD range (mean OD of 1,9 for both species) as illustrated in Figure 4-6 and the results were considered valid. An acceptable coefficient of variance of 8% was, nonetheless, attained for the equine control serum.

After exclusion of the two invalid bovine control values for each of the protein A/G: HRP and the anti-bovine IgG: HRP, the respective coefficients of variance were 23% and 24%. The large variation of the means of the bovine controls across all the plates, as illustrated by the frequency distributions, may partly be attributed to testing being done in different laboratories with intervals of several months. Differences in substrate development timing were observed, which is considered the reason for the large variation in the bovine control. Assuming a homologues control sample across all plates it was, however, not considered to impact the study outcomes, as all results were interpreted in relation to the controls on each respective plate. Although the wide range of control bovine serum values should not be problematic in this particular study design, variation in the substrate development time may impede on repeatability of results and scientific merit. It resulted in uncertainty regarding the suitability of homologous bovine serum as a control and may require consideration of purified bovine IgG as control for a more refined study design, particularly when results should be related to a mean control value.

Kelly et al. (1993) reported that horse, goat, buffalo, wildebeest, waterbuck and impala reacted slightly stronger to the recombinant protein A/G than protein A. Furthermore,

elephant, rhinoceros and giraffe bound much stronger to protein A/G and pig reacted equally well to both conjugates. In this study, however, rhinoceros, zebra, warthog and buffalo out-performed elephant and giraffe (results not valid) by far. Iimpala barely rose from the zero line in the UI, whereas both steenbok and duiker reacted strongly, although this may be due to clustering and small sample size and has to be verified.

Feir et al. (1993), assessed protein A and recombinant protein G instead of using secondary Ab from closely related domestic animal species in an immunodiffusion assay. Of note is the confirmation that impala, that did not react with either protein A or recombinant protein G, also did not bind to protein A/G in this study. They also reported that sable, kudu and springbok reacted almost solely to Protein G and black rhinoceros reacted better to protein A (Feir et al., 1993). Sable and springbok both reacted comparable to the bovine control and black rhinoceros performed far superior to that to the recombinant protein A/G. Kudu, on the other hand, did not bind at all to the protein A/G:HRP in this study.

Stöbel et al. (2002) assessed reactivity to conjugated protein A or protein G and found that protein G bound stronger in most ungulate species, confirming the finding of Kelly et al. (1993) and Feir et al. (1993). The exceptions were blesbok (medium binding with protein G), and kudu (medium to both protein A and protein G and contradicted in this study for protein A/G: HRP), while impala (again confirming the results of this study), nyala, gemsbok (oryx), roan (all three comparable with this study) and buffalo (in contrast to this study) achieved only weak binding with protein G. They also reported that the rhinoceros species all tended to react stronger to protein G, which is contradictory to the earlier findings of Feir et al. (1993), but supports the finding by Kelly et al. (1993) with stronger affinity to

protein A/G in these species and was also confirmed in this study. Their finding that zebra, springbok and warthog bound strongly with both protein A and protein G was also confirmed by this study, as was elephant, which bound to a medium extent only with protein A (Stöbel et al., 2002).

In their study, Kramsky et al. (2003a) similarly compared the binding of purified wildlife IgG to recombinant protein G with the binding capacity of purified bovine IgG as a positive control. The published results for three overlapping species, namely sable, binding equivalently to cattle, impala and oryx (gemsbok), binding significantly less than cattle, were confirmed by this study, but there is a notable difference in the results for kudu, nyala and roan, which were reported to bind to protein G equivalent to cattle, with lower reactivity with protein A/G: HRP compared to the bovine control in this study (Kramsky et al., 2003a).

Kelly et al. (1993), Feir et al. (1993), Stöbel et al. (2002) and Kramsky et al. (2003a) also reported that kudu bound with either protein G or protein A+G, which renders the result for kudu, not binding with the recombinant protein A/G: HRP in this study, the most striking contrasting finding, and should be investigated further. In this study, where the kudu serum samples consistently bound significantly lower with all three conjugates compared to the control sera, retesting to confirm the results will be recommended. Should this result be reproduced, thereby ruling out a problem with this set of samples, the possibility of a genetically linked predisposition that renders the recombinant protein A/G: HRP molecule to bind significantly less with the IgG of at least some kudu subpopulations could also be considered. As protein A has five binding sites, protein G has two and the recombinant protein A/G: HRP has six known binding sites on the Fc-portion (ThermoScientific, 2016),

one may speculate that a binding site lost during recombinant protein expression might be the binding site on protein G that kudu binds with. This is probably a matter for further investigation.

The consistent finding of weak cross-reactivity of impala to all the conjugates by several authors and confirmed in this study is also of note. It may be of value to also consider an anti-goat or anti-sheep conjugate for this species.

It should also be noted that the results for duiker are based on only two available serum samples (n=2). The implied limited variance may be due to clustering as the two samples originated from the same source. In order to quantify intra-species variation more accurately, these findings should definitely be confirmed by a larger and more representative sample. The intraspecies variation was more evident in the steenbok (n=3) results, with considerably wider confidence intervals.

Eight wildlife species, namely Burchell's zebra, white rhinoceros, black rhinoceros, common warthog, steenbok, African buffalo, duiker and sable, performed better than or equal to the bovine control with the recombinant protein A/G: HRP. On the usefulness index (UI) (Figure 4-13) five other species, namely elephant, hippopotamus, tsessebe, blesbok and springbok preformed slightly lower than the control with UI value above 1, all with a t-value >-3 and p-value ≥0,05. As there was not enough evidence to reject the null hypothesis for the difference in cross-reactivity from the control, they were also considered equivalent to the bovine control. The black and blue wildebeest as well as gemsbok also had a UI value above 1, but the respective t-values of -3,8 -3, 9 and -4,9 were in these cases highly significant ($p<0.05$), the null hypothesis was rejected and the difference from the

control accepted. All the other species performed significantly lower than the bovine control (UI below zero). Recombinant protein A/G: HRP was therefore considered to be useful for 13 of the tested wildlife species.

No wildlife species even reached a value of 1 on the UI graph for the rabbit anti-bovine IgG: HRP used in this study and apart from steenbok (n=3) the t-values were consistently below -8 with highly significant p-values. The rabbit anti-bovine IgG: HRP was not found to be useful for any of the wildlife species tested in this study.

Reading the UI graph for the goat anti-horse IgG: HRP, however, Hartmann's mountain zebra, Burchell's zebra and black rhinoceros performed on par with or slightly better than the equine control (p-values >0,1). White rhinoceros on the zero line, with a t-value of -2,4 and p-value ≥0,05 was also found equivalent to the control. The goat anti-horse IgG: HRP could potentially be useful in these four species, but the recombinant protein A/G: HRP performed comparably better in all four these species.

Sample size could potentially be influenced by intra-species variation for a specific candidate conjugate. In this study, intra-species variation was more pronounced for the protein A/G: HRP and it should be considered when evaluating candidate conjugates or for validation of assays making use of cross-reactive conjugates. This is of particular importance if generalisation of results are attempted for application in local populations and conditions.

Due to variation in cross-reactivity and functional avidity of the different species, it is essential to properly validate a test for a specific species and calculate appropriate cut-off

values to ensure that appropriate test sensitivity and specificity levels are reached and the results can be interpreted accurately.

Other polyclonal conjugates may perform better than some of those tested in this study. It is proposed that the sum of the optical density and the relative avidity index ratios be used as an indication of the possible usefulness of a candidate conjugate. It is, however, postulated, that if such a usefulness index is appropriately described and influential parameters or indicators, such as the t-value of the two-sided t-test, are quantified, it could become a useful instrument in the search for appropriate conjugates for wildlife species, which also may benefit other wildlife classes such as carnivores and various other small mammal orders as well.

The sum distribution is often used in the case of dependent variables, whereas the product distribution is more applicable to independent variables. Although a reasonable assumption is that binding and affinity is probably dependent variables, the inter-variable relationship has not been established as part of this study. As these two variables may be conditionally independent the product distribution could be investigated as an alternative calculation for the usefulness index.

In summary, this study found that recombinant protein A/G: HRP may be useful for concideration as a potential ELISA conjugate for 13 tested African herbivore and hoof stock wildlife species. This study also proposed a *usefulness index* (UI), which is a quantitative rating tool calculated from the binding capacity (OD) and binding strength (RAI) of candidate enzyme conjugated molecules with wildlife serum IgG related to a known homologous control. It makes provision for objective interpretation of the UI by using the

t-value and p-value of the 2-sided t-test. The summation UI for the three tested conjugates is demonstrated as a bar chart in Figure 5-1 below.

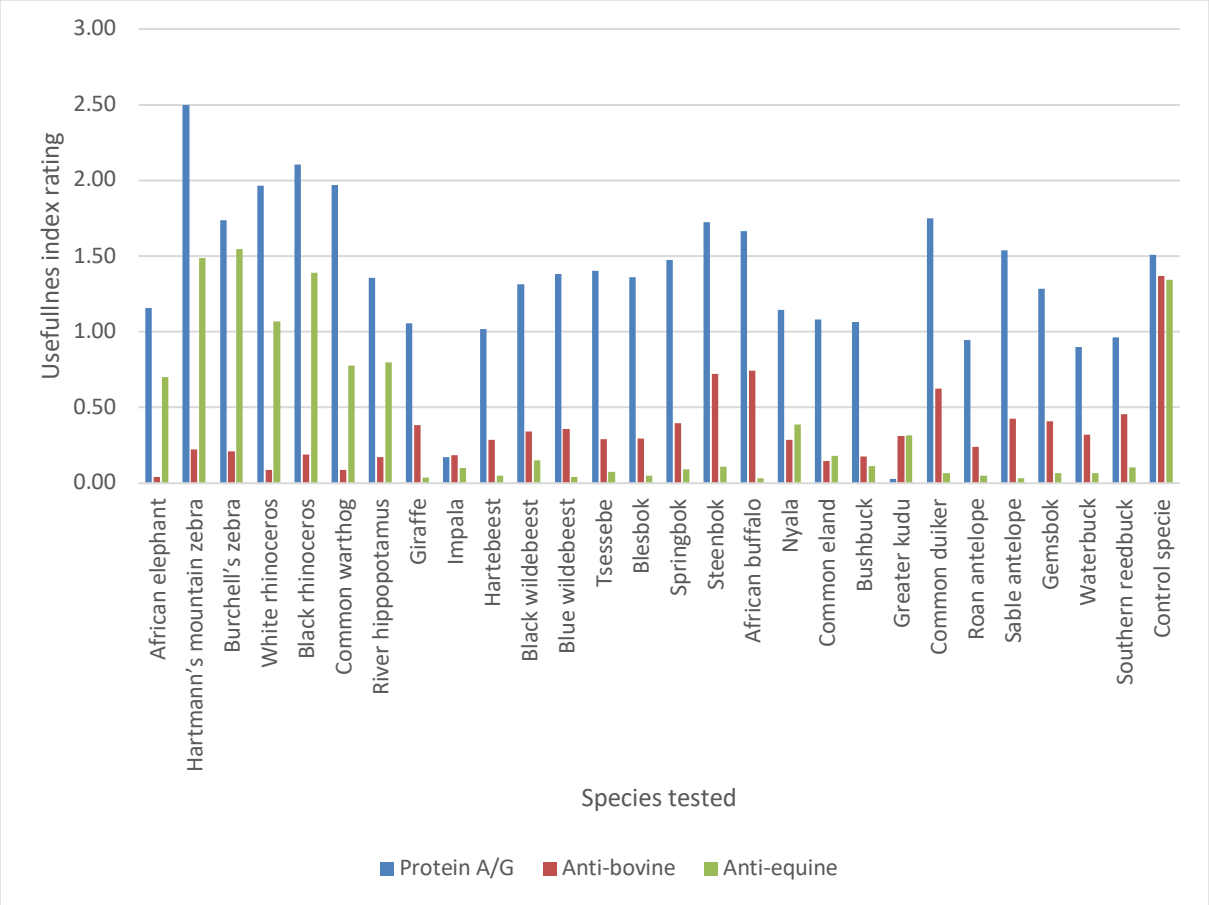

Figure 5-1: Bar chart of the of usefulness index for the three conjugates using the sum of the sample to positive (SP) ratio of the ODs and the RAI ratios to rate the conjugates. Invalid bovine controls on the recombinant protein A/G: HRP and rabbit anti-bovine IgG: HRP plates for Hartmann's mountain zebra and giraffe resulted in data that cannot be validated.

5.1 References

ABCAM 2010. Understanding secondary antibodies. *In:* ABCAM (ed.).

ABDELGAWAD, A., HERMES, R., DAMIANI, A., LAMGLAIT, B., CZIRJÁK, G. Á., EAST, M., ASCHENBORN, O., WENKER, C., KASEM, S., OSTERRIEDER, N. & GREENWOOD, A. D. 2015. Comprehensive Serology Based on a Peptide ELISA to Assess the Prevalence of Closely Related Equine Herpesviruses in Zoo and Wild Animals. *PLoS ONE,* 10**,** e0138370.

ABUTARBUSH, S. M. 2008. Saunders Comprehensive Veterinary Dictionary, 3rd ed. *The Canadian Veterinary Journal,* 49**,** 906-906.

AFSHAR, A., THOMAS, F. C., WRIGHT, P. F., SHAPIRO, J. L., SHETTIGARA, P. T. & ANDERSON, J. 1987. Comparison of competitive and indirect enzyme-linked immunosorbent assays for detection of bluetongue virus antibodies in serum and whole blood. *J Clin Microbiol,* 25**,** 1705-10.

ALBERGHINA, D., FAZIO, F., ARFUSO, F., SCIANO, S., ZUMBO, A. & PICCIONE, G. 2013. Reference Intervals of Serum Protein Concentrations from Clinically Healthy Female Ragusana Donkeys (Equus asinus) Determined by Cellulose Acetate Electrophoresis. *Journal of Equine Veterinary Science,* 33**,** 433-436.

ALBERGHINA, D., GIANNETTO, C., VAZZANA, I., FERRANTELLI, V. & PICCIONE, G. 2011. Reference intervals for total protein concentration, serum protein fractions, and albumin/globulin ratios in clinically healthy dairy cows. *Journal of Veterinary Diagnostic Investigation,* 23**,** 111-114.

ALLSOPP, B., BABIUK, L. & BABIUK, S. 2004. Vaccination: an approach to the control of infectious diseases. *Infectious Diseases of Livestock***,** 239-247.

ALMANZAR, G., OTTENSMEIER, B., LIESE, J. & PRELOG, M. 2013. Assessment of IgG avidity against pertussis toxin and filamentous hemagglutinin via an adapted enzyme-linked immunosorbent assay (ELISA) using ammonium thiocyanate. *Journal of Immunological Methods,* 387**,** 36-42.

ALPHA DIAGNOSTIC, I. 2017. Rabbit Anti-Deer IgG (H+L chain) (Cervid family: Deer, Elk, Moose)-Biotin Conjugate. Online: Alpha Diagnostic International Ltd.

ANDERSON, J. 1984. Use of monoclonal antibody in a blocking ELISA to detect group specific antibodies to bluetongue virus. *J Immunol Methods,* 74**,** 139-49.

BARNARD, B. J. 1997. Antibodies against some viruses of domestic animals in southern African wild animals. *Onderstepoort J Vet Res,* 64**,** 95-110.

BARNARD, B. J. & PAWESKA, J. T. 1993. Prevalence of antibodies against some equine viruses in zebra (Zebra burchelli) in the Kruger National Park, 1991-1992. *Onderstepoort J Vet Res,* 60**,** 175-9.

BENGIS, R., KOCK, R., THOMSON, G. & BIGALKE, R. 2004. Infectious diseases of animals in sub-Saharan Africa: The wildlife/livestock interface. *Infectious diseases of livestock,* 2.

BIO-RAD. 2017. *Helpful ELISA Hints* [Online]. Online: Bio-Rad. Available: https://www.bio-rad-antibodies.com/helpful-elisa-hints.html [Accessed 14 July 2017 2017].

BLACKBURN, N. & SWANEPOEL, R. 1988. Observations on antibody levels associated with active and passive immunity to African horse sickness. *Tropical animal health and production,* 20**,** 203-210.

DAUNER, J. G., PAN, Y., HILDESHEIM, A., KEMP, T. J., PORRAS, C. & PINTO, L. A. 2012. Development and application of a GuHCl-modified ELISA to measure the avidity of anti-HPV L1 VLP antibodies in vaccinated individuals. *Molecular and Cellular Probes,* 26**,** 73-80.

DAVIES, F. G., OTIENO, S. & JESSETT, D. M. 1977. The antibody response in sheep vaccinated with experimental Nairobi sheep disease vaccines. *Tropical Animal Health and Production,* 9**,** 181-183.

DIMITROV, J. D., LACROIX-DESMAZES, S. & KAVERI, S. V. 2011. Important parameters for evaluation of antibody avidity by immunosorbent assay. *Analytical Biochemistry,* 418**,** 149-151.

ERASMUS, B. 1978. A new approach to polyvalent immunization against African horsesickness. *Journal of Equine Medicine and Surgery. Supplement*.

EVANS, A., GAKUYA, F., PAWESKA, J. T., ROSTAL, M., AKOOLO, L., VAN VUREN, P. J., MANYIBE, T., MACHARIA, J. M., KSIAZEK, T. G., FEIKIN, D. R., BREIMAN, R. F. & KARIUKI NJENGA, M. 2008. Prevalence of antibodies against Rift Valley fever virus in Kenyan wildlife. *Epidemiology and infection,* 136**,** 1261-9.

FEIR, D., LAU, C. & JUNGE, R. 1993. Protein A and protein G in the diagnosis of diseases in zoo animals. *Transactions of the Missouri Academy of Science,* 27**,** 9-14.

FERREIRA, M. U. & KATZIN, A. M. 1995. The assessment of antibody affinity distribution by thiocyanate elution: a simple dose-response approach. *Journal of Immunological Methods,* 187**,** 297-305.

FISCHER-TENHAGEN, C., HAMBLIN, C., QUANDT, S. & FROLICH, K. 2000. Serosurvey for selected infectious disease agents in free-ranging black and white rhinoceros in Africa. *J Wildl Dis,* 36**,** 316-23.

GABORICK, C. M., SALMAN, M. D., ELLIS, R. P. & TRIANTIS, J. 1996. Evaluation of a five-antigen ELISA for diagnosis of tuberculosis in cattle and Cervidae. *J Am Vet Med Assoc,* 209**,** 962-6.

GARDNER, I. A., HIETALA, S. & BOYCE, W. M. 1996. Validity of using serological tests for diagnosis of diseases in wild animals. *Rev Sci Tech,* 15.

GERDES, G. 2004. A South African overview of the virus, vectors, surveillance and unique features of bluetongue. *Vet Ital,* 40**,** 39-42.

GLASER, R. W. 1993. Determination of antibody affinity by ELISA with a non-linear regression program: Evaluation of linearized approximations. *Journal of Immunological Methods,* 160**,** 129-133.

HAJELA, K. 1991. Structure and function of Fc receptors. *Biochemical education,* 19**,** 50-57.

HAMBLIN, C., BURNETT, I. T. R. & HEDGER, R. S. 1986. A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus. I. Development and method of ELISA. *Journal of Immunological Methods,* 93**,** 115-121.

HUDSON, L. & HAY, F. C. 1989. *Practical Immunology,* Great Britain, The Bath Press.

JENNER, E. 1800. *An inquiry into the causes and effects of the variolae vaccinae, a disease discovered in some of the western counties of England, particularly Gloucestershire, and known by the name of the cow pox*, author.

KELLY, P. J., TAGWIRA, M., MATTHEWMAN, L., MASON, P. R. & WRIGHT, E. P. 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.

KINDT, T. J., GOLDSBY, R. A. & OSBORNE, B. A. 2007. Kuby immunology. New York: WH Freeman.

KNEITZ, R.-H., SCHUBERT, J., TOLLMANN, F., ZENS, W., HEDMAN, K. & WEISSBRICH, B. 2004. A new method for determination of varicella-zoster virus immunoglobulin G avidity in serum and cerebrospinal fluid. *BMC infectious diseases,* 4**,** 33.

KOHLER, G. & MILSTEIN, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature,* 256**,** 495-497.

KRAMSKY, J. A., MANNING, E. J. & COLLINS, M. T. 2003a. Protein G binding to enriched serum immunoglobulin from nondomestic hoofstock species. *Journal of veterinary diagnostic investigation,* 15**,** 253-261.

KRAMSKY, J. A., MANNING, E. J. B. & COLLINS, M. T. 2003b. Protein G binding to enriched serum immunoglobulin from nondomestic hoofstock species. *J Vet Diagn Invest,* 15.

LARSEN, R. S., SALMAN, M. D., MIKOTA, S. K., ISAZA, R., MONTALI, R. J. & TRIANTIS, J. 2000. Evaluation of a multiple-antigen enzyme-linked immunosorbent assay for detection of Mycobacterium tuberculosis infection in captive elephants. *J Zoo Wildl Med,* 31**,** 291-302.

LEQUIN, R. M. 2005. Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). *Clin Chem,* 51**,** 2415-8.

LINDEQUE, P., BRAIN, C., VERSFELD, W. & TURNBULL, P. 1996. Anthrax vaccine-induced seroconversion in zebra and elephant in the Etosha National Park, Namibia. *Salisbury Med Bull,* 87**,** 113-5.

LIPMAN, N. S., JACKSON, L. R., TRUDEL, L. J. & WEIS-GARCIA, F. 2005. Monoclonal Versus Polyclonal Antibodies: Distinguishing Characteristics, Applications, and Information Resources. *ILAR Journal,* 46**,** 258-268.

LORCA-ORÓ, C., LÓPEZ-OLVERA, J. R., RUIZ-FONS, F., ACEVEDO, P., GARCÍA-BOCANEGRA, I., OLEAGA, Á., GORTÁZAR, C. & PUJOLS, J. 2014. Long-Term Dynamics of Bluetongue Virus in Wild Ruminants: Relationship with Outbreaks in Livestock in Spain, 2006-2011. *PLOS ONE,* 9**,** e100027.

MACDONALD, R. A., HOSKING, C. S. & JONES, C. L. 1988. The measurement of relative antibody affinity by ELISA using thiocyanate elution. *Journal of Immunological Methods,* 106**,** 191-194.

MEROC, E., HERR, C., VERHEYDEN, B., HOOYBERGHS, J., HOUDART, P., RAEMAEKERS, M., VANDENBUSSCHE, F., DE CLERCQ, K. & MINTIENS, K. 2009. Bluetongue in Belgium: episode II. *Transboundary and emerging diseases,* 56**,** 39-48.

MILLER, M., BUSS, P., JOUBERT, J., MASEKO, N., HOFMEYR, M. & GERDES, T. 2011. Serosurvey for selected viral agents in white rhinoceros (Ceratotherium simum) in Kruger National Park, 2007. *J Zoo Wildl Med,* 42**,** 29-32.

NAGY, O., TOTHOVA, C., NAGYOVA, V. & KOVAC, G. 2015. Comparison of serum protein electrophoretic pattern in cows and small ruminants. *Acta Veterinaria Brno,* 84**,** 187-195.

NOBEL, M. 2014. *The Nobel Prize in Physiology or Medicine 1984* [Online]. Nobelprize.org: Nobel Media AB. Available: http://www.nobelprize.org/nobel_prizes/medicine/laureates/1984/ [Accessed 15 Feb 2017].

OIE 2017. Manual of Diagnostic Tests and Vaccines

for Terrestrial Animals 2017. Online: OIE.

PAWESKA, J. T., SMITH, S. J., WRIGHT, I. M., WILLIAMS, R., COHEN, A. S., VAN DIJK, A. A., GROBBELAAR, A. A., CROFT, J. E., SWANEPOEL, R. & GERDES, G. H. 2003. Indirect enzyme-linked immunosorbent assay for the detection of antibody against Rift Valley fever virus in domestic and wild ruminant sera. *The Onderstepoort journal of veterinary research,* 70**,** 49-64.

PRUVOT, M., FORDE, T. L., STEELE, J., KUTZ, S. J., BUCK, J. D., MEER, F. V. D. & ORSEL, K. 2013. The modification and evaluation of an ELISA test for the surveillance of Mycobacterium avium subsp. paratuberculosisinfection in wild ruminants. *BMC Veterinary Research,* 9**,** 5.

ROSSI, S., PIOZ, M., BEARD, E., DURAND, B., GIBERT, P., GAUTHIER, D., KLEIN, F., MAILLARD, D., SAINT-ANDRIEUX, C. & SAUBUSSE, T. 2014. Bluetongue dynamics in French wildlife: exploring the driving forces. *Transboundary and emerging diseases,* 61.

RYAN, K. & RAY, C. 2004. Sherris medical microbiology: an introduction to infectious diseases. 4th. McGraw Hall USA.

SIGMA-ALDRICH. 2017. *Secondary Antibodies, Conjugates and Kits* [Online]. http://www.sigmaaldrich.com: Sigma-Aldrich. Available: http://www.sigmaaldrich.com [Accessed 16 February 2017 2017].

SKINNER, J. D. & CHIMIMBA, C. T. 2005. *The Mammals of the Southern African Sub-region*, Cambridge University Press.

SMITH, J. E., CHAVEY, P. S. & MILLER, R. E. 1995. Iron Metabolism in Captive Black (Diceros bicornis) and White (Ceratotherium simum) Rhinoceroses. *Journal of Zoo and Wildlife Medicine,* 26**,** 525-531.

STEYN, J., VENTER, G., COETZEE, P. & VENTER, E. 2015. The epidemiology of bluetongue virus in Mnisi, South Africa. *American Journal of Epidemiology and Infectious Disease,* 3**,** 95-102.

STÖBEL, K., SCHONBERG, A. & STAAK, C. 2002. A new non-species dependent ELISA for detection of antibodies to Borrelia burgdorferi s. l. in zoo animals. *Int J Med Microbiol,* 291 Suppl 33**,** 88-99.

THERMOFISHERSCIENTIFIC 2010. ELISA technical guide and protocols. *In:* INCORPORATE, T. F. S. (ed.). Online: Thermo Fisher Scientific Incorporate.

THERMOFISHERSCIENTIFIC. 2017. *Comparison of Antibody IGg Binding Proteins* [Online]. Antibody Resource Library: ThermoFisher Scientific. Available: https://www.thermofisher.com/za/en/home/life-science/antibodies/antibodies-learning-center/antibodies-resource-library/antibody-methods/comparison-antibody-igg-binding-proteins.html [Accessed 6 July 2017 2017].

THERMOSCIENTIFIC 2016. Binding characteristics of antibody-binding proteins: Protein A, Protein G, Protein A/G and Protein L. *In:* THERMOSCIENTIFIC (ed.). Online: ThermoScientific.

THEVASAGAYAM, J. A., WOOLHOUSE, T. R., MERTENS, P. P., BURROUGHS, J. N. & ANDERSON, J. 1996. Monoclonal antibody based competitive ELISA for the detection of antibodies against epizootic haemorrhagic disease of deer virus. *J Virol Methods,* 57**,** 117-26.

THRUSFIELD, M. 2013. *Veterinary epidemiology,* Great Brittain, Elsevier.

TIZARD, I. R. 2013. *Veterinary immunology,* St. Louis, Mo. :, Elsevier/Saunders.

TURNBULL, P. C. B., BROSTER, M. G., CARMAN, J. A., MANCHEE, R. J. & MELLING, J. 1986. Development of antibodies to protective antigen and lethal factor components of anthrax toxin in humans and guinea pigs and their relevance to protective immunity. *Infection and Immunity,* 52**,** 356-363.

TURNBULL, P. C. B., TINDALL, B. W., COETZEE, J. D., CONRADIE, C. M., BULL, R. L., LINDEQUE, P. M. & HUEBSCHLE, O. J. B. 2004. Vaccine-induced protection against anthrax in cheetah (Acinonyx jubatus) and black rhinoceros (Diceros bicornis). *Vaccine,* 22**,** 3340-3347.

VAN OSS, C., GOOD, R. & CHAUDHURY, M. 1986. Nature of the antigen-antibody interaction: Primary and secondary bonds: optimal conditions for association and dissociation. *Journal of Chromatography B: Biomedical Sciences and Applications,* 376**,** 111-119.

WASHINGTON, J. A. 1996. Principles of Diagnosis: Serodiagnosis in: Baron's Medical Microbiology. *In:* BARON, S. (ed.) *Medical Microbiology.* Fourth ed. Galveston, Texas: Univ of Texas Medical Branch.

Chapter 6 Conclusion

The purpose of this study was to evaluate the cross-reactivity of commercially available polyvalent anti-bovine IgG: HRP, anti-horse IgG: HRP and binding of recombinant protein A/G: HRP with serum IgG of various African herbivore wildlife species.

Thirteen wildlife species performed better than or equal to the bovine control with the recombinant protein A/G: HRP while the rest of the species performed significantly lower than the bovine control. In contrast, the rabbit anti-bovine IgG: HRP was found not to be useful for any of the wildlife species tested in this study. The goat anti-horse IgG: HRP was found to be equivalent to the equine control in four species, while the recombinant protein A/G: HRP performed better in all four these species.

The calculation of a usefulness index using the optical density (as a ratio of a known homologues control value) and the relative avidity index (a ratio expressing binding strength) could become a useful tool for evaluating and comparing the cross-reactivity between anti-species conjugates and serum from heterologous species. Although the inter-variable relationship has not been established as part of this study, it is assumed that molecular binding and affinity are probably two dependant variables. The sum distribution is often used in the case of dependant variables, whereas the product distribution is more applicable to independent variables. Should these two variables be found to be conditionally independent, the product distribution may be more appropriate and should be investigated as an alternative usefulness index formula.

There is strong agreement with previously published findings and some identified research opportunities to explain unexpected results were identified, while this study made some contribution to the knowledge regarding Southern African herbivore wildlife species.

Appendix 1. AEC Certificates

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**The testing of various commercially available conjugates for cross reactivity to serum from various wildlife species using ELISA assay**
PROJECT NUMBER	**V086-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. A Cloete**

STUDENT NUMBER (where applicable)	**UP_86454422**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Buffalo, kudu, sitatunga, njala, bosbok, eland, hartebeest, wildebeest, blesbok, bontebok, tsessebe, sable, roan, gemsbok, reedbok, puku, letchewe, waterbuck, impala, pringbok, gryskok, steenbok, ribbok, dik-dikkie, klipspringer, suni, oribi, duikers, tahr**	**Deer types, Hippopotamus, giraffe, pigs**	**Elephant, rhinoceros, zebra, dassies, aardvark**
NUMBER OF SAMPLES	**10**	**10**	10
Approval period to use animals for research/testing purposes			**July 2016-July 2017**
SUPERVISOR	**Dr. J Crafford**		

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 25 July 2016
CHAIRMAN: UP Animal Ethics Committee	Signature

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

Extension No. 1

PROJECT TITLE	**The testing of various commercially available conjugates for cross reactivity to serum from various wildlife species using ELISA assay**
PROJECT NUMBER	**V086-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. A Cloete**

STUDENT NUMBER (where applicable)	**UP_86454422**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SAMPLES (Stored Serum)	**Buffalo, kudu, sitatunga, njala, bosbok, eland, hartebeest, wildebeest, blesbok, bontebok, tsessebe, sable, roan, gemsbok, reedbok, puku, letchewe, waterbuck, impala, pringbok, gryskok, steenbok, ribbok, dik-dikkie, klipspringer, suni, oribi, duikers, tahr**	**Deer types, Hippopotamus, giraffe, pigs**	**Elephant, rhinoceros, zebra, dassies, aardvark**
NUMBER OF SAMPLES	**10**	**10**	**10**
Approval period to use animals for research/testing purposes			**Jan. 2017-Jan 2018**
SUPERVISOR	**Dr. J Crafford**		

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 15 March 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

Appendix 2. ELISA data

1. Effect of the chaotrope on the conjugate

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.715	0.467	1.563	1.505	0.333	0.335	1.373	1.489	1.484	1.445	0.243	0.251
B	1.245	1.033	1.511	1.524	0.324	0.329	1.408	1.587	1.478	1.381	0.202	0.21
C	1.486	1.443	1.519	1.509	0.304	0.314	1.378	1.542	1.449	1.405	0.153	0.173
D	1.581	1.49	1.563	1.481	0.303	0.32	1.364	1.491	1.479	1.324	0.114	0.122
E	1.542	1.506	1.494	1.449	0.342	0.321	1.377	1.532	1.469	1.346	0.097	0.096
F	1.53	1.508	1.461	1.431	0.401	0.386	1.356	1.512	1.414	1.284	0.082	0.092
G	1.545	1.554	1.479	1.453	0.595	0.582	1.338	1.547	1.425	1.496	0.063	0.066
H	1.533	1.456	1.386	1.435	1.334	1.276	1.258	1.499	1.425	1.3	0.052	0.053

2. Checker-board titration to determine serum and conjugate dilution

a. Bovine serum and Protein A/G: HRP

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.340	1.020	0.654	0.413	0.267	0.155	0.103	0.077	0.062	0.050	0.048	0.048
B	1.591	1.317	1.061	0.744	0.414	0.231	0.157	0.099	0.065	0.060	0.046	0.043
C	1.658	1.398	1.063	0.770	0.471	0.291	0.159	0.100	0.076	0.059	0.050	0.044
D	1.624	1.434	1.256	0.882	0.467	0.316	0.162	0.119	0.085	0.065	0.052	0.044
E	1.703	1.460	1.193	0.803	0.515	0.320	0.175	0.121	0.087	0.063	0.056	0.047
F	0.863	1.293	0.995	0.643	0.389	0.261	0.165	0.111	0,079	0.057	0.051	0.047
G	0085	1.107	0.826	0.503	0.278	0.211	0.354	0.108	0.067	0.542	0.050	0.045
H	0.046	0.041	0.041	0.043	0.042	0.044	0.042	0.043	0.044	0.036	0.045	0.045

b. Bovine serum and anti-bovine IgG: HRP

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.976	0.565	0.358	0.254	0.194	0.120	0.082	0.082	0.082	0.069	0.530	0.055
B	0.584	0.331	0.218	0.114	0.102	0.077	0.464	0.042	0.044	0.039	0.046	0.042
C	0.649	0.309	0.219	0.104	0.233	0.063	0.053	0.047	0.045	0.044	0.042	0.040
D	0.631	0.221	0.133	0.072	0.057	0.053	0.054	0.046	0.043	0.045	0.044	0.040
E	0.913	0.265	0.146	0.256	0.376	0.212	0.469	0.044	0.415	0.045	0.045	0.043
F	0.477	0.219	0.166	0.098	0.533	0.053	0.050	0.045	0.044	0.043	0.045	0.229
G	0.491	0.176	0.113	0.076	0.059	0.051	0.458	0.046	0.048	0.044	0.040	0.048
H	0.040	0.039	0.039	00.043	0.040	0.047	0.050	0.043	0.038	0.042	0.042	0.043

c. Equine serum and anti-horse IgG: HRP

	1	2	3	4	5	6	7	8	9	10	*11	*12
A	3.058	3.117	3.124	3.042	2.903	2.556	2.473	2.386	1.985	1.462	3.000	2.854
B	2.414	2.377	2.164	2.192	1.822	1.748	1.683	1.609	1.343	1.192	2.247	2.051
C	1.698	1.472	1.448	1.515	1.307	1.343	1.240	1.049	0.999	0.622	1.526	1.948
D	0.983	1.169	1.103	1.032	0.867	0.878	0.824	0.723	0.653	0.449	1.108	1.236
E	0.548	0.567	0.59	0.523	0.442	0.453	0.378	0.44	0.401	0.236	1.804	2.115
F	0.33	0.279	0.31	0.297	0.298	0.248	0.294	0.269	0.223	0.145	1.267	1.988
G	0.198	0.208	0.222	0.19	0.156	0.169	0.158	0.147	0.144	0.121	1.492	1.612
H	0.152	0.128	0.149	0.153	0.341	0.244	0.139	0.132	0.114	0.089	1.201	1.549

*Columns 11 and 12 on this plate were used for another purpose and do not form part of the anti-horse checker board titration

d. Chaotrope titration for protein A/G: HRP: Titrations of protein A/G with 1:2000 bovine, black wildebeest and gemsbok serum

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.056	0.498	0.061	0.561	0.055	0.58	0.059	0.601	0.049	0.403	0.054	0.373
B	0.057	0.524	0.059	0.496	0.058	0.594	0.06	0.535	0.05	0.365	0.052	0.369
C	0.061	0.48	0.067	0.415	0.071	0.504	0.066	0.468	0.052	0.367	0.398	0.08
D	0.074	0.393	0.08	0.38	0.084	0.44	0.08	0.437	0.073	0.411	0.069	0.33
E	0.131	0.563	0.137	0.418	0.124	0.498	0.111	0.466	0.099	0.356	0.1	0.39
F	0.174	0.426	0.212	0.43	0.178	0.48	0.191	0.452	0.113	0.343	0.152	0.397
G	0.271	0.481	0.306	0.449	0.257	0.482	0.277	0.453	0.179	0.371	0.232	0.384
H	0.396	0.472	0.48	0.518	0.407	0.549	0.421	0.516	0.273	0.339	0.332	0.349

e. Chaotrope titration for rabbit anti-bovine IgG: HRP: Titrations of anti-bovine IgG: HRP with 1:2000 bovine, black wildebeest and gemsbok serum

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.039	0.207	0.043	0.206	0.043	0.07	0.04	0.073	0.046	0.072	0.041	0.096
B	0.042	0.045	0.044	0.208	0.042	0.062	0.038	0.059	0.042	0.066	0.041	0.08
C	0.041	0.19	0.044	0.197	0.04	0.066	0.043	0.062	0.043	0.066	0.04	0.08
D	0.045	0.224	0.042	0.193	0.04	0.055	0.039	0.061	0.041	0.066	0.039	0.081
E	0.046	0.194	0.047	0.205	0.039	0.058	0.037	0.061	0.039	0.064	0.041	0.078
F	0.055	0.177	0.05	0.191	0.042	0.061	0.038	0.06	0.036	0.061	0.042	0.074
G	0.071	0.184	0.072	0.199	0.042	0.063	0.041	0.056	0.041	0.063	0.043	0.085
H	0.115	0.185	0.1	0.204	0.048	0.065	0.047	0.061	0.049	0.076	0.05	0.093

3. Testing wildlife species

For all species, the order of the results below are anti-bovine IgG: HRP, followed by Protein A/G: HRP and thirdly anti-horse IgG: HRP. Cells (wells) in red were excluded from calculation due to contamination in the laboratory (mistakes and spillages).

(1) African elephant

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.042	0.04	0.043	0.047	0.041	0.045	0.047	0.048	0.048	0.051	0.969	1.242
F	0.044	0.039	0.047	0.049	0.043	0.044	0.045	0.045	0.044	0.049	1.085	1.164
G	0.038	0.039	0.038	0.04	0.038	0.042	0.042	0.04	0.041	0.04	0.347	0.315
H	0.036	0.038	0.038	0.041	0.038	0.04	0.04	0.039	0.041	0.042	0.345	0.267

	1	2	3	4	5	6	7	8	9	10	11	12
E	2.302	1.606	2.063	1.965	2.355	1.887	1.577	2.003	1.532	2.289	2.014	2.191
F	2.278	1.734	2.228	1.875	2.13	1.653	1.669	2.005	1.537	2.391	2.025	2.032
G	0.456	0.265	0.423	0.401	0.567	0.3	0.312	0.526	0.242	0.751	0.809	0.764
H	0.393	0.311	0.476	0.349	0.433	0.255	0.3	0.48	0.253	0.792	0.906	0.758

	1	2	3	4	5	6	7	8	9	10	11	12
E	1.419	1.195	1.253	1.296	1.481	1.186	1.145	1.499	1.288	1.321	2.026	2.076
F	1.33	1.185	1.297	1.271	1.389	1.196	1.268	1.376	1.309	1.406	2.114	2.099
G	0.09	0.098	0.083	0.077	0.096	0.084	0.088	0.099	0.091	0.097	0.297	0.269
H	0.08	0.1	0.098	0.083	0.104	0.08	0.09	0.114	0.082	0.122	0.318	0.334

(2) Mountain zebra (Hartmann's)

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.213	0.098	0.08	0.114	0.114	0.104	0.081	0.075	0.084	0.096	0.401	0.561
F	0.17	0.068	0.092	0.1	0.092	0.14	0.105	0.111	0.099	0.096	0.462	0.474
G	0.075	0.048	0.045	0.051	0.05	0.067	0.051	0.044	0.049	0.049	0.238	0.215
H	0.074	0.05	0.037	0.051	0.051	0.055	0.047	0.048	0.049	0.055	0.249	0.232

	1	2	3	4	5	6	7	8	9	10	11	12
E	1.077	0.497	0.529	0.844	0.686	0.417	0.493	0.936	0.677	0.753	0.401	0.509
F	0.795	0.597	0.681	0.922	0.673	0.569	0.514	0.947	0.709	0.728	0.353	0.388
G	0.722	0.447	0.489	0.781	0.555	0.35	0.442	0.6	0.605	0.606	0.417	0.3
H	0.653	0.444	0.417	0.753	0.662	0.366	0.431	0.685	0.546	0.639	0.431	0.378

	1	2	3	4	5	6	7	8	9	10	11	12
E	1.989	1.999	1.876	1.743	1.948	1.337	1.668	1.644	1.778	1.856	1.912	1.823
F	1.928	1.941	1.847	1.912	1.871	1.704	1.65	1.846	1.854	1.901	1.925	2.013

G	1.2	1.006	0.915	1.211	1.044	0.869	0.961	0.987	0.877	1.039	1.21	1.06
H	1.128	0.919	0.82	1.237	0.957	0.77	0.909	0.892	1.043	0.924	1.153	0.845

(3) Burchell's zebra

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.137	0.146	0.128	0.162	0.098	0.173	0.335	0.114	0.144	0.522	0.665	0.67
F	0.128	0.117	0.113	0.167	0.129	0.131	0.107	0.086	0.118	0.1	0.604	0.653
G	0.049	0.048	0.044	0.045	0.16	0.045	0.046	0.043	0.045	0.043	0.216	0.189
H	0.055	0.059	0.049	0.048	0.045	0.046	0.051	0.048	0.042	0.04	0.245	0.233

	1	2	3	4	5	6	7	8	9	10	11	12
E	1.269	1.007	1.225	1.399	1.203	1.507	1.267	1.122	1.339	1.189	1.123	1.157
F	1.204	1.109	1.107	1.279	0.849	1.187	1.103	1.025	1.236	1.223	1.068	1.023
G	0.737	0.895	0.843	0.901	0.813	0.954	0.775	0.716	0.796	0.665	0.62	0.458
H	0.682	0.673	0.648	0.665	0.775	0.809	0.743	0.7	0.931	0.703	0.654	0.543

	1	2	3	4	5	6	7	8	9	10	11	12
E	1.964	2.025	1.933	1.971	1.986	2.208	1.817	1.848	1.917	1.911	1.902	1.913
F	1.951	1.998	1.889	1.975	1.807	2.311	1.851	1.731	1.784	1.907	1.813	1.748
G	1.02	1.02	0.931	0.936	0.937	1.028	1.148	0.947	1.023	1.004	1.06	1.141
H	1.039	0.937	0.959	0.854	0.908	0.966	0.937	0.838	0.817	0.981	1.063	1.128

(4) White rhinoceros

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.456	0.273	0.07	0.063	0.081	0.07	0.071	0.071	0.083	0.089	0.905	0.945
B	0.44	0.058	0.054	0.059	0.074	0.066	0.064	0.06	0.066	0.068	0.876	1.008
C	0.084	0.045	0.042	0.036	0.037	0.049	0.046	0.043	0.044	0.036	0.256	0.28
D	0.088	0.042	0.041	0.039	0.042	0.043	0.037	0.043	0.042	0.044	0.289	0.29

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.435	2.416	2.342	2.424	2.457	2.43	2.337	2.399	2.451	2.389	2.209	2.232
B	2.437	2.481	2.411	2.407	2.344	2.356	2.278	2.307	2.386	2.41	2.097	2.288
C	1.526	2.148	2.192	2.211	2.19	2.387	2.269	2.183	2.039	2.164	0.734	0.669
D	1.392	2.068	2.189	2.181	2.138	2.2	2.108	2.278	1.993	2.239	0.736	0.739

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.913	1.984	2.011	1.984	1.892	1.922	1.991	1.972	1.996	1.897	2.084	2.08
B	2.251	2.012	2.182	1.989	2.088	2.062	2.183	2.129	2.133	2.024	2.288	2.162
C	0.343	0.302	0.228	0.272	0.251	0.256	0.239	0.244	0.241	0.234	0.306	0.44
D	0.262	0.224	0.264	0.257	0.207	0.239	0.247	0.268	0.312	0.251	0.339	0.428

(5) Black rhinoceros

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.095	0.151	0.154	0.163	0.177	0.097	0.15	0.155	0.139	0.12	0.765	0.639
B	0.111	0.145	0.128	0.125	0.103	0.128	0.108	0.11	0.112	0.113	0.706	0.62
C	0.045	0.044	0.047	0.044	0.045	0.046	0.046	0.046	0.044	0.043	0.259	0.233
D	0.04	0.057	0.043	0.046	0.044	0.045	0.048	0.047	0.048	0.05	0.226	0.267

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.641	1.907	1.626	1.883	1.488	1.705	1.348	1.274	1.391	1.473	1.121	1.134
B	1.54	1.686	1.376	1.686	1.417	1.476	1.365	1.333	1.38	1.481	1.106	1.008
C	1.143	1.276	1.242	1.469	1.239	0.886	0.892	1.071	0.982	0.87	0.462	0.395
D	1.264	1.073	0.912	1.12	1.228	1.213	1.1	0.875	0.749	0.993	0.509	0.45

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.951	1.934	1.932	1.905	1.923	1.895	1.784	1.857	1.891	1.992	1.903	1.917
B	1.959	2.031	1.854	1.964	1.877	1.855	1.7	1.895	1.911	1.826	1.841	2.081
C	0.836	0.927	0.879	0.836	0.821	0.863	0.822	0.669	0.784	0.775	0.987	1.366
D	0.737	0.724	0.64	0.769	0.791	0.746	0.827	0.662	0.689	0.722	1.1	1.125

(6) Common warthog

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.13	0.082	0.075	0.071	0.095	0.099	0.084	0.07	0.08	0.072	0.955	0.999
F	0.14	0.085	0.08	0.067	0.09	0.09	0.085	0.073	0.08	0.075	0.956	0.981
G	0.044	0.044	0.046	0.042	0.047	0.047	0.047	0.041	0.042	0.041	0.364	0.332
H	0.044	0.049	0.048	0.046	0.048	0.044	0.045	0.043	0.055	0.048	0.393	0.343

4	1	2	3	4	5	6	7	8	9	10	11	12
E	2.471	2.436	2.355	2.379	2.425	2.372	2.466	2.48	2.521	2.403	2.221	2.196
F	2.491	2.373	2.495	2.381	2.332	2.261	2.35	2.406	2.537	2.365	2.14	2.132
G	2.13	2.106	1.993	1.968	2.108	2.033	1.932	1.798	2.199	1.88	1.008	0.876
H	2.114	2.128	2.174	1.828	2.303	2.299	2.277	1.963	2.215	1.927	1.074	0.926

	1	2	3	4	5	6	7	8	9	10	11	12
E	1.741	1.746	1.441	1.623	1.648	1.689	1.591	1.38	1.516	1.425	2.273	2.224
F	1.786	1.763	1.477	1.574	1.631	1.623	1.676	1.408	1.401	1.483	2.44	2.237
G	0.153	0.148	0.103	0.141	0.173	0.136	0.123	0.131	0.114	0.139	0.59	0.421
H	0.141	0.16	0.129	0.126	0.158	0.173	0.124	0.098	0.138	0.12	0.439	0.401

(7) River hippopotamus

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.258	0.203	0.323	0.17	0.298	0.353	0.351	0.28	0.354	0.413	1.488	1.38
B	0.172	0.143	0.141	0.147	0.165	0.144	0.223	0.138	0.192	0.143	1.203	1.321
C	0.053	0.056	0.058	0.062	0.065	0.058	0.072	0.06	0.058	0.061	0.375	0.316
D	0.051	0.053	0.057	0.06	0.059	0.056	0.072	0.056	0.058	0.059	0.357	0.359

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.339	2.097	1.962	2.109	1.978	1.92	2.148	1.821	1.716	2.09	2.035	2.229
B	2.309	2.183	1.991	1.958	1.917	2.028	2.03	1.941	1.815	2.117	2.009	2.056
C	0.883	0.897	0.79	0.807	0.71	0.798	0.973	0.558	0.617	0.773	0.843	0.786
D	0.833	0.787	0.703	0.816	0.723	0.821	1.097	0.586	0.604	0.738	0.83	0.817

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.586	1.55	1.525	1.425	1.42	1.464	1.242	1.5	1.629	1.402	1.852	1.913
B	1.542	1.351	1.334	1.4	1.211	1.6	1.255	1.574	1.311	1.509	2.174	2.21
C	0.14	0.125	0.116	0.115	0.182	0.154	0.163	0.197	0.112	0.128	0.284	0.302
D	0.15	0.093	0.137	0.097	0.105	0.141	0.112	0.131	0.116	0.118	0.293	0.337

(8) Giraffe

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.236	0.271	0.187	0.163	0.155	0.284	0.289	0.287	0.254	0.245	0.546	0.501
B	0.206	0.201	0.176	0.155	0.086	0.182	0.105	0.157	0.144	0.144	0.473	0.532
C	0.07	0.079	0.083	0.056	0.055	0.07	0.066	0.058	0.063	0.057	0.237	0.259
D	0.072	0.075	0.077	0.058	0.047	0.064	0.055	0.056	0.073	0.06	0.173	0.179

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.6	0.579	0.612	0.18	0.221	0.389	0.324	0.356	0.5	0.347	0.605	0.706
B	0.734	0.442	0.534	0.155	0.137	0.419	0.237	0.284	0.372	0.405	0.55	0.653
C	0.372	0.189	0.289	0.094	0.076	0.141	0.099	0.135	0.203	0.15	0.402	0.416
D	0.269	0.205	0.236	0.081	0.067	0.14	0.094	0.136	0.232	0.18	0.417	0.367

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.067	0.43	0.086	0.059	0.064	0.074	0.084	0.082	0.063	0.086	1.905	1.962
B	0.073	0.058	0.059	0.061	0.054	0.079	0.064	0.054	0.045	0.065	1.809	1.954
C	0.034	0.032	0.038	0.033	0.039	0.042	0.04	0.041	0.033	0.045	1.284	1.091
D	0.034	0.04	0.033	0.043	0.043	0.044	0.037	0.042	0.04	0.042	1.191	1.262

(9) Impala

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.219	0.163	0.214	0.194	0.149	0.146	0.273	0.201	0.16	0.215	1.025	1.056
F	0.295	0.165	0.205	0.19	0.433	OV	0.393	0.212	0.187	0.213	1.178	1.19
G	1.497	0.077	0.086	0.158	1.899	2.515	0.28	0.138	0.101	0.085	0.416	0.369
H	0.105	0.068	0.124	0.799	2.744	0.493	0.083	0.297	0.145	0.083	0.413	0.353

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.109	0.152	0.504	0.249	0.159	0.211	0.414	0.548	0.204	0.447	1.621	1.712
F	0.118	0.122	0.446	0.221	0.17	0.17	0.352	0.5	0.175	0.46	1.638	1.609
G	0.059	0.078	0.176	0.097	0.084	0.093	0.15	0.194	0.084	0.159	0.948	0.761
H	0.062	0.077	0.163	0.095	0.092	0.088	0.622	0.178	0.088	0.171	0.843	0.721

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.137	0.127	0.322	0.183	0.12	0.12	0.212	0.478	0.207	0.362	2.232	2.118
F	0.154	0.128	0.294	0.169	0.113	0.127	0.228	0.48	0.195	0.339	2.63	1.927
G	0.056	0.052	0.054	0.056	0.051	0.054	0.055	0.067	0.055	0.052	0.511	0.431
H	0.064	0.051	0.08	0.057	0.06	0.062	0.159	0.427	0.058	0.053	0.415	0.387

Ov=overflow

(10) Hartebeest

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.231	0.501	0.22	0.195	0.285	0.283	0.266	0.236	0.207	0.269	0.864	0.913
B	0.194	0.296	0.261	0.171	0.224	0.255	0.224	0.194	0.191	0.209	0.804	0.852
C	0.082	0.108	0.094	0.075	0.088	0.086	0.08	0.078	0.07	0.08	0.37	0.413
D	0.092	0.106	0.092	0.063	0.085	0.092	0.085	0.074	0.071	0.079	0.329	0.347

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.776	1.076	0.944	0.785	0.962	1.052	1.182	0.737	0.791	0.932	1.334	1.303
B	0.638	0.976	0.649	0.717	0.747	1.185	0.868	0.716	0.712	0.824	1.174	1.277
C	0.163	0.492	0.291	0.278	0.402	0.434	0.348	0.287	0.179	0.294	0.765	0.61
D	0.163	0.392	0.224	0.189	0.356	0.365	0.319	0.251	0.145	0.268	0.558	0.632

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.094	0.087	0.174	0.09	0.08	0.149	0.096	0.083	0.257	0.096	1.969	2.027
B	0.075	0.064	0.151	0.065	0.061	0.121	0.055	0.065	0.08	0.074	1.995	2.045
C	0.046	0.043	0.051	0.047	0.045	0.045	0.052	0.044	0.046	0.05	1.027	1.284
D	0.048	0.044	0.057	0.043	0.045	0.051	0.049	0.045	0.047	0.045	1.132	1.123

(11) Black wildebeest

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.235	0.229	0.177	0.244	0.127	0.272	0.233	0.253	0.247	0.273	0.666	0.658
F	0.248	0.232	0.191	0.225	0.115	0.251	0.25	0.235	0.236	0.296	0.611	0.737
G	0.091	0.105	0.072	0.096	0.045	0.086	0.087	0.066	0.08	0.092	0.342	0.333
H	0.107	0.071	0.065	0.076	0.05	0.101	0.124	0.069	0.084	0.09	0.311	0.306

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.972	0.839	0.704	0.675	0.048	0.807	1.347	0.491	1.143	0.685	1.305	1.504
F	0.801	0.765	0.725	0.596	0.047	0.48	1.356	0.46	1.051	0.959	1.099	1.216
G	0.552	0.473	0.471	0.469	0.044	0.568	0.902	0.289	0.729	0.891	0.945	0.961
H	0.602	0.521	0.508	0.382	0.074	0.482	1.053	0.297	0.79	0.84	0.638	0.837

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.539	0.445	0.256	0.224	0.06	0.174	0.52	0.099	0.154	0.33	1.889	1.838
F	0.485	0.548	0.297	0.239	0.055	0.205	0.572	0.088	0.133	0.347	2.008	1.844
G	0.14	0.134	0.07	0.074	0.043	0.06	0.13	0.06	0.051	0.07	1.21	1.072
H	0.108	0.116	0.055	0.058	0.042	0.052	0.111	0.041	0.048	0.069	1.226	1.101

(12) Blue wildebeest

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.801	0.435	0.579	0.43	0.428	0.258	0.329	0.28	0.373	0.563	1.155	1.17
B	0.768	0.558	0.395	0.246	0.224	0.211	0.219	0.252	0.255	0.211	1.046	0.994
C	0.165	0.122	0.156	0.137	0.105	0.114	0.114	0.119	0.131	0.126	0.457	0.418
D	0.178	0.117	0.11	0.115	0.116	0.098	0.113	0.101	0.094	0.093	0.42	0.335

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.066	1.566	1.743	1.977	1.538	1.518	1.663	1.815	1.475	1.683	1.837	2.079
B	2.046	1.425	1.679	1.751	1.504	1.37	1.421	1.844	1.335	1.517	1.98	2.059
C	1.147	0.782	0.847	1.168	0.825	0.746	0.891	1.109	0.74	0.787	1.26	1.276
D	1.113	0.82	0.803	1.174	0.795	0.771	1.033	1.231	0.696	0.733	1.31	1.189

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.188	0.162	0.089	0.113	0.104	0.107	0.093	0.098	0.066	0.066	2.668	2.356
B	0.184	0.083	0.094	0.085	0.075	0.091	0.074	0.092	0.079	0.059	2.285	2.17
C	0.311	0.06	0.056	0.062	0.052	0.059	0.097	0.065	0.051	0.049	0.822	0.671
D	0.11	0.061	0.053	0.051	0.058	0.069	0.052	0.139	0.053	0.078	0.779	0.589

(13) Tsessebe

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.891	0.885	0.269	0.328	0.306	0.434	0.275	0.328	0.226	0.314	0.288	0.271
B	0.854	0.923	0.242	0.174	0.152	0.29	0.178	0.221	0.156	0.192	0.194	0.292
C	0.433	0.392	0.097	0.085	0.08	0.11	0.091	0.111	0.06	0.081	0.089	0.075
D	0.415	0.462	0.092	0.086	0.094	0.118	0.092	0.101	0.089	0.078	0.091	0.084

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.711	0.827	0.886	0.653	0.956	0.778	1.466	0.845	0.892	0.746	1.034	1.26
F	0.824	0.913	0.978	0.671	0.904	0.805	1.034	0.865	0.714	0.949	0.938	1.087
G	0.396	0.572	0.53	0.456	0.647	0.479	0.611	0.459	0.424	0.431	0.655	0.627
H	0.404	0.647	0.642	0.469	0.603	0.445	0.756	0.515	0.405	0.474	0.69	0.461

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.104	0.094	0.112	0.105	0.16	0.765	0.125	0.13	0.123	0.117	1.964	1.941
F	0.074	0.101	0.095	0.086	0.153	0.102	0.125	0.116	0.1	0.109	1.901	1.88
G	0.04	0.046	0.046	0.044	0.05	0.046	0.047	0.04	0.047	0.048	1.154	0.908
H	0.04	0.063	0.045	0.053	0.051	0.048	0.045	0.038	0.054	0.046	1.091	0.858

(14) Blesbok

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.27	0.2	0.301	0.258	0.297	0.339	0.717	1.133	0.202	0.242	1.091	1.149
B	0.209	0.234	0.264	0.28	0.259	0.198	0.341	0.309	0.198	0.212	1.055	1.069
C	0.078	0.086	0.081	0.105	0.106	0.085	0.182	0.097	0.087	0.085	0.425	0.365
D	0.082	0.1	0.08	0.1	0.088	0.101	0.139	0.089	0.088	0.082	0.367	0.363

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.671	1.469	1.75	2.104	3.572	1.877	1.886	1.435	1.592	2.09	2.157	1.848
B	0.686	1.752	1.576	1.825	1.993	1.676	1.68	1.28	1.453	1.563	1.814	1.819
C	0.24	0.839	0.494	0.736	0.961	1.046	0.974	0.554	0.891	1.304	1.155	0.96
D	0.22	1.052	0.796	0.909	0.871	0.809	0.978	0.577	0.683	1.124	0.975	0.79

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.153	0.176	0.119	0.128	0.173	0.152	0.124	0.161	0.094	0.093	2.313	2.299
B	0.152	0.123	0.112	0.092	0.098	0.098	0.126	0.083	0.077	0.077	2.178	2.96
C	0.077	0.055	0.051	0.051	0.051	0.058	0.058	0.049	0.048	0.05	0.646	0.891
D	0.115	0.094	0.049	0.058	0.055	0.054	0.054	0.05	0.051	0.05	0.604	0.588

(15) Springbok

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.911	0.526	0.378	0.234	0.381	0.295	0.243	0.274	0.204	0.286	0.98	1.002
F	1.048	0.483	0.346	0.277	0.613	2.496	0.292	0.293	0.268	0.257	0.917	0.98
G	0.421	0.201	0.278	0.644	3.998	OF	0.257	0.17	0.122	0.165	0.448	0.422
H	0.374	1.514	1.194	3.189	OF	2.662	0.703	0.64	0.586	0.136	0.462	0.532

	1	2	3	4	5	6	7	8	9	10	11	12
E	2.236	1.52	0.892	1.049	1.792	1.535	2.032	1.647	1.498	1.956	1.806	2.077
F	2.316	1.573	0.852	1.008	1.854	1.198	1.619	1.695	2.289	1.855	1.822	1.944
G	1.886	0.892	0.37	0.546	1.165	0.637	1.094	OF	1.566	1.12	1.218	1.107
H	1.966	0.763	0.367	0.556	1.185	0.852	2.88	OF	0.928	1.266	1.151	1.189

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.537	0.173	0.096	0.111	0.225	0.109	0.139	0.162	0.119	0.228	2.389	2.477
F	1.068	0.168	0.079	0.104	0.22	0.102	0.268	0.172	0.116	0.216	2.369	2.322
G	0.101	0.06	0.05	0.048	0.242	3.752	0.703	0.066	0.048	0.049	0.674	0.57
H	0.072	0.055	0.052	0.088	0.645	OF	0.469	0.065	0.047	0.047	0.446	0.422

OF = overflow (spillage/contamination)

(16) Steenbok

Only 3 animals (samples) available

9	1	2	3	4	5	6	7	8	9	10	11	12
A	0.589	0.978	0.528	0.28	0.041	0.038	0.043	0.045	0.051	0.331	1.165	1.077
B	0.537	1.017	0.504	0.322	0.042	0.048	0.047	0.036	0.046	0.405	1.77	1.026
C	0.103	0.186	0.088	0.067	0.036	0.072	0.057	0.058	0.045	0.056	0.293	0.315
D	0.064	0.181	0.091	0.057	0.037	0.064	0.069	0.064	0.041	0.046	0.268	0.233

10	1	2	3	4	5	6	7	8	9	10	11	12
A	2.017	2.298	1.911	0.074	0.044	0.046	0.044	0.039	0.041	0.141	1.658	1.728
B	1.984	2.34	1.854	0.051	0.032	0.045	0.034	0.035	0.035	0.149	1.807	1.531
C	0.757	1.649	0.635	0.038	0.033	0.031	0.034	0.036	0.034	0.044	0.647	0.507
D	0.736	1.722	0.658	0.043	0.038	0.036	0.039	0.039	0.039	0.044	0.538	0.459

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.426	0.109	0.132	0.055	0.057	0.033	0.031	0.031	0.031	0.032	2.128	2.032
B	0.485	0.095	0.123	0.05	0.06	0.038	0.033	0.03	0.03	0.048	2.187	2.175
C	0.054	0.042	0.045	0.041	0.04	0.034	0.031	0.127	0.035	0.03	0.345	0.346
D	0.049	0.038	0.043	0.042	0.039	0.03	0.031	0.033	0.037	0.03	0.327	0.424

Only 3 animals tested

(17) African buffalo

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.599	0.631	0.204	0.551	0.66	0.525	0.468	0.428	0.455	0.608	0.937	1.092
F	0.701	0.672	0.203	0.529	0.731	0.514	0.466	0.466	0.512	0.615	1.051	1.068
G	0.144	0.175	0.069	0.136	0.167	0.135	0.122	0.117	0.124	0.145	0.322	0.304
H	0.136	0.158	0.07	0.131	0.156	0.12	0.119	0.102	0.121	0.145	0.318	0.303

	1	2	3	4	5	6	7	8	9	10	11	12
E	2.411	2.354	1.683	2.424	2.409	2.408	2.352	2.414	2.44	2.359	2.303	2.221
F	2.483	2.328	1.692	2.336	2.373	2.271	2.33	2.382	2.281	2.397	2.171	2.223
G	1.463	1.575	0.616	1.483	1.742	1.714	1.42	1.679	1.457	1.781	0.853	0.758
H	1.288	1.568	0.622	1.335	1.719	1.639	1.427	1.615	1.336	1.699	0.721	0.659

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.135	0.062	0.101	0.103	0.054	0.058	0.052	0.048	0.052	0.05	2.263	2.312
F	0.128	0.073	0.126	0.095	0.053	0.057	0.052	0.052	0.057	0.049	2.176	2.196
G	0.043	0.044	0.044	0.042	0.041	0.042	0.042	0.041	0.04	0.048	0.452	0.342
H	0.043	0.046	0.043	0.047	0.048	0.043	0.045	0.042	0.045	0.052	0.411	0.384

Red cells: discard results due to procedure error (only 9 animals for anti-bovine IgG: HRP)

(18) Nyala

The blue wells in the table above is the CT treatment and the black values are the PBS (swopped)

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.174	0.335	0.184	0.239	0.225	0.36	0.124	0.255	0.196	0.157	0.716	0.775
F	0.183	0.338	0.207	0.148	0.184	0.302	0.12	0.307	0.181	0.185	0.767	0.823
G	0.068	0.105	0.06	0.063	0.069	0.094	0.063	0.094	0.078	0.066	0.364	0.377
H	0.076	0.11	0.072	0.058	0.069	0.101	0.064	0.094	0.073	0.061	0.362	0.361

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.579	1.456	0.683	0.568	0.794	1.19	0.402	1.384	0.819	0.443	1.277	1.375
F	0.534	1.55	0.632	0.525	0.757	1.371	0.376	1.419	0.94	0.593	1.051	1.341
G	0.176	0.791	0.285	0.228	0.264	0.768	0.211	0.619	0.314	0.155	0.709	0.651
H	0.175	0.878	0.281	0.207	0.282	0.856	0.219	0.696	0.369	0.207	0.798	0.572

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.104	0.229	0.124	0.105	0.132	0.239	0.063	0.122	0.087	0.069	1.124	1.05
F	0.108	0.267	0.148	0.112	0.142	0.275	0.071	0.163	0.085	0.061	1.129	1.017
G	0.601	1.183	0.839	0.589	0.736	1.241	0.355	0.967	1.612	0.369	2.018	1.983
H	0.586	1.207	0.702	0.514	0.774	1.206	0.326	0.878	0.552	0.325	2.01	2.03

(19) Common eland

	1	2	3	4	5	6	7	8	9	10	11	12
E	1.48	0.279	0.351	0.189	0.14	0.235	1.475	0.14	0.137	0.104	1.052	1.099
F	0.672	0.324	0.455	0.193	0.148	0.175	0.186	0.157	0.167	0.115	1.071	1.198
G	0.191	0.08	0.141	0.083	0.069	0.176	0.429	OF	0.268	0.064	0.482	0.417
H	0.155	0.139	0.135	0.084	0.081	0.371	3.58	2.303	0.075	0.057	0.369	0.372

	1	2	3	4	5	6	7	8	9	10	11	12
E	1.462	0.928	1.965	1.217	1.041	0.777	0.889	0.887	0.812	0.791	1.668	1.828
F	1.293	0.792	2.063	1.165	0.848	0.765	0.66	1.012	0.804	0.63	1.68	1.669
G	0.47	0.342	1.37	0.621	0.345	0.28	0.347	0.413	0.35	0.307	1.052	0.921
H	0.579	0.275	1.354	0.646	0.411	0.312	0.376	0.405	0.296	0.312	0.891	0.84

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.437	0.242	0.978	0.483	0.424	0.184	0.275	0.437	0.359	0.15	2.281	2.271
F	0.408	0.263	0.911	0.452	0.363	0.186	0.289	0.561	0.402	0.165	2.32	2.021
G	0.078	0.064	0.143	0.065	0.852	0.091	0.075	0.061	0.053	0.05	0.453	0.432
H	0.081	0.097	0.067	0.065	0.065	0.087	0.061	0.056	0.05	0.053	0.388	0.391

(20) Bushbuck

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.303	0.32	0.205	0.149	0.24	0.206	0.16	0.17	0.17	0.14	1.252	1.078
B	0.294	0.21	0.182	0.123	0.207	0.172	0.184	0.157	0.168	0.137	1.018	1.023
C	0.081	0.077	0.072	0.059	0.082	0.082	0.067	0.072	0.072	0.063	0.494	0.343
D	0.075	0.081	0.083	0.064	0.084	0.079	0.072	0.067	0.069	0.065	0.404	0.327

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.089	2.096	1.989	1.723	2.133	2.089	1.956	1.813	1.454	1.52	2.226	2.284
B	2.024	2.083	1.882	1.458	2.034	2.107	2.003	1.784	1.682	1.392	2.379	2.329
C	0.393	0.482	0.419	0.268	0.7	0.593	0.555	0.458	0.378	0.32	1.344	1.039
D	0.442	0.457	0.514	0.318	0.73	0.566	0.627	0.595	0.396	0.379	1.118	1.099

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.398	0.165	0.169	0.146	0.175	0.341	0.34	0.294	0.172	0.233	1.943	2.096
B	0.381	0.181	0.176	0.134	0.174	0.297	0.342	0.361	0.15	0.223	2.428	2.268
C	0.051	0.044	0.044	0.044	0.05	0.079	0.047	0.043	0.044	0.212	0.357	0.316
D	0.048	0.041	0.076	0.048	0.055	0.045	0.047	0.052	0.044	0.043	0.355	0.403

(21) Greater kudu

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.28	0.136	0.232	0.289	0.374	0.326	0.296	0.408	0.371	0.463	1.087	1.224
B	0.415	0.298	0.363	0.429	0.23	0.309	0.445	0.654	0.7	0.485	1.281	1.237
C	0.04	0.042	0.045	0.039	0.043	0.045	0.043	0.042	0.044	0.043	0.507	0.352
D	0.042	0.041	0.045	0.045	0.044	0.048	0.053	0.049	0.048	0.047	0.484	0.378

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.048	0.041	0.038	0.047	0.038	0.116	0.043	0.063	0.038	0.056	1.796	2.047
B	0.033	0.04	0.042	0.036	0.045	0.049	0.04	0.043	0.042	0.17	1.864	2.015
C	0.032	0.04	0.039	0.038	0.037	0.041	0.037	0.041	0.04	0.042	0.764	0.846
D	0.034	0.043	0.041	0.034	0.033	0.039	0.043	0.043	0.053	0.04	0.888	1.035

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.718	0.52	0.484	0.502	0.613	0.751	0.654	0.256	1.397	0.485	1.976	1.943
B	0.795	0.535	0.5	0.541	0.676	0.772	0.682	0.286	1.351	0.475	2.162	2.153
C	0.089	0.061	0.057	0.056	0.069	0.083	0.07	0.056	0.17	0.06	0.52	0.491
D	0.072	0.052	0.06	0.059	0.071	0.084	0.099	0.046	0.207	0.077	0.638	0.634

(22) Common duiker

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.563	0.494	0.261	0.284	0.039	0.051	0.053	0.056	0.039	0.312	1.118	1.171
F	0.546	0.493	0.325	0.293	0.035	0.045	0.051	0.048	0.047	0.235	1.018	1.05
G	0.068	0.081	0.046	0.041	0.035	0.047	0.045	0.054	0.045	0.045	0.27	0.196
H	0.083	0.068	0.04	0.037	0.037	0.053	0.067	0.04	0.037	0.044	0.216	0.198

	1	2	3	4	5	6	7	8	9	10	11	12
E	2.07	2.056	0.117	0.036	0.034	0.036	0.033	0.031	0.038	0.034	1.38	1.598
F	1.994	1.876	0.103	0.053	0.075	0.032	0.038	0.03	0.038	0.042	1.425	1.464
G	0.898	0.725	0.042	0.036	0.031	0.052	0.031	0.033	0.032	0.039	0.455	0.462
H	0.777	0.704	0.038	0.047	0.033	0.034	0.058	0.032	0.039	0.04	0.43	0.404

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.187	0.109	0.051	0.059	0.059	0.033	0.031	0.035	0.045	0.04	2.121	2.155
F	0.165	0.095	0.041	0.049	0.043	0.03	0.03	0.031	0.029	0.028	2.096	2.144
G	0.041	0.041	0.039	0.04	0.038	0.03	0.033	0.03	0.027	0.025	0.405	0.411
H	0.043	0.04	0.046	0.049	0.038	0.029	0.03	0.03	0.03	0.031	0.387	0.345

Only two serum samples available and tested

(23) Roan antelope

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.77	0.857	0.151	0.205	0.203	0.244	0.154	0.146	0.207	0.136	0.229	0.238
F	0.855	0.872	0.175	0.216	0.218	0.279	0.164	0.155	0.219	0.151	0.252	0.263
G	0.426	0.379	0.08	0.094	0.095	0.116	0.081	0.075	0.107	0.108	0.185	0.1
H	0.382	0.442	0.076	0.09	0.098	0.135	0.082	0.075	0.094	0.081	0.124	0.126

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.609	0.593	0.441	0.689	0.289	0.448	0.82	0.447	0.561	0.376	1.372	1.36
B	0.466	0.507	0.387	0.683	0.241	0.335	0.69	0.413	0.418	0.301	1.115	1.31
C	0.28	0.286	0.253	0.507	0.153	0.203	0.491	0.251	0.263	0.157	0.677	0.651
D	0.239	0.286	0.192	0.476	0.134	0.196	0.488	0.25	0.257	0.17	0.583	0.548

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.073	0.108	0.104	0.08	0.106	0.08	0.052	0.124	0.125	0.082	1.932	1.958
B	0.103	0.139	0.098	0.091	0.127	0.102	0.063	0.101	0.087	0.146	2.01	1.905
C	0.057	0.057	0.049	0.045	0.045	0.057	0.044	0.046	0.045	0.055	1.272	0.971
D	0.05	0.049	0.045	0.048	0.045	0.051	0.044	0.043	0.053	0.043	1.033	0.967

(24) Sable antelope

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.473	0.346	0.594	0.587	0.623	0.637	0.674	0.431	0.59	0.517	1.322	1.32
F	0.54	0.409	0.636	0.478	0.522	0.807	0.644	0.443	0.73	0.481	1.277	1.307
G	0.107	0.089	0.084	0.089	0.1	0.092	0.125	0.093	0.233	0.111	0.428	0.377
H	0.102	0.068	0.082	0.084	0.088	0.087	0.117	0.079	0.257	0.084	0.399	0.315

	1	2	3	4	5	6	7	8	9	10	11	12
E	2.314	2.134	1.947	1.76	1.854	1.819	2.188	1.811	2.319	1.662	1.951	2.047
F	2.307	2.168	1.934	1.721	2.036	2.023	2.207	1.774	2.313	1.737	1.883	2.148
G	1.529	1.072	0.82	0.8	0.847	0.97	1.213	0.673	2.293	0.613	0.969	0.996
H	1.527	1.098	1.152	0.74	0.894	0.836	1.149	0.593	2.173	0.59	0.916	0.925

	1	2	3	4	5	6	7	8	9	10	11	12
E	0.051	0.079	0.05	0.048	0.042	0.045	0.059	0.04	0.109	0.06	2.172	2.167
F	0.057	0.045	0.044	0.053	0.046	0.043	0.059	0.307	0.063	0.062	2.217	2.017
G	0.041	0.043	0.039	0.04	0.04	0.046	0.042	0.096	0.044	0.049	0.605	0.482
H	0.041	0.048	0.041	0.042	0.04	0.041	0.042	0.043	0.348	0.056	0.625	0.54

(25) Gemsbok

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.821	1.07	0.679	0.607	0.621	0.43	0.188	0.317	0.946	0.599	1.244	1.182
B	0.785	0.706	0.475	0.313	0.443	0.284	0.147	0.196	0.252	0.363	1.14	1.115
C	0.276	0.157	0.152	0.13	0.221	0.167	0.077	0.123	0.13	0.187	0.49	0.389
D	0.967	0.237	0.137	0.138	0.21	0.471	0.156	0.123	0.131	0.175	0.462	0.429

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.277	1.547	1.247	0.968	1.671	2.675	3.119	1.016	1.447	1.327	1.836	1.8
B	1.073	1.323	1.12	0.631	1.12	1.084	0.245	0.683	1.068	1.256	1.76	1.744
C	1.049	0.847	0.672	0.383	1.083	0.697	0.114	0.525	0.924	0.802	1.211	1.169
D	0.992	0.628	0.6	0.408	1.016	0.435	0.119	0.493	0.804	0.808	1.132	0.929

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.057	0.053	0.054	0.039	0.053	0.198	0.052	0.05	0.047	0.05	0.874	0.78
B	0.118	0.174	0.071	0.068	0.08	0.541	0.069	0.075	0.085	0.11	2.207	2.355
C	0.049	0.05	0.053	0.051	0.053	0.058	0.047	0.046	0.044	0.049	0.26	0.354
D	0.051	0.046	0.048	0.049	0.061	0.068	0.053	0.048	0.051	0.057	0.485	0.423

(26) Waterbuck

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.189	0.184	0.337	0.265	0.257	0.271	0.217	0.241	0.19	0.22	0.664	0.688
B	0.126	0.234	0.21	0.183	0.234	0.215	0.138	0.24	0.211	0.188	0.64	0.716
C	0.051	0.063	0.08	0.061	0.067	0.062	0.054	0.057	0.063	0.063	0.315	0.31
D	0.062	0.061	0.089	0.06	0.057	0.058	0.054	0.083	0.06	0.067	0.237	0.276

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.392	0.528	0.955	0.399	0.857	0.405	0.366	0.48	0.64	0.986	1.471	1.377
B	0.518	0.395	0.884	0.348	0.687	0.38	0.24	0.447	0.513	0.685	1.178	1.54
C	0.285	0.128	0.445	0.251	0.501	0.191	0.155	0.236	0.325	0.411	0.842	0.819
D	0.222	0.157	0.376	0.216	0.444	0.192	0.135	0.241	0.241	0.398	0.798	0.761

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.104	0.142	0.13	0.116	0.23	0.12	0.155	0.129	0.126	0.136	1.882	1.924
B	0.128	0.11	0.138	0.104	0.218	0.12	0.12	0.099	0.086	0.104	1.967	2.055
C	0.048	0.047	0.047	0.044	0.053	0.066	0.074	0.054	0.034	0.047	1.035	0.994
D	0.064	0.051	0.046	0.056	0.052	0.048	0.051	0.047	0.045	0.046	1.231	0.9

(27) Southern reedbuck

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.725	0.772	0.839	0.305	0.258	0.814	0.304	0.064	0.071	0.156	1.084	1.113
B	0.921	0.751	0.486	0.256	0.188	0.234	0.137	0.057	0.047	0.049	1.08	1.107
C	0.177	0.097	0.148	0.103	0.107	0.113	0.07	0.045	0.049	0.055	0.47	0.425
D	0.142	0.086	0.148	0.098	0.111	0.108	0.064	0.047	0.048	0.044	0.425	0.346

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.451	0.765	1.994	2.465	3.922	1.355	0.752	0.262	0.257	0.316	2.04	1.786
B	1.357	0.686	1.674	2.13	2.041	3.758	0.503	0.095	0.102	0.1	1.711	1.823
C	0.693	0.298	1.026	0.95	1.439	1.05	0.21	0.048	0.051	0.05	1.064	0.991
D	0.644	0.252	0.903	0.824	1.367	0.977	0.163	0.051	0.047	0.075	0.989	0.952

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.206	0.134	0.837	0.28	0.225	0.172	0.113	0.069	0.074	0.072	2.345	2.485
B	0.219	0.082	0.74	0.189	0.174	0.096	0.065	0.066	0.055	0.052	2.437	2.41
C	0.057	0.048	0.082	0.059	0.071	0.091	0.059	0.056	0.05	0.09	0.77	0.61
D	0.052	0.058	0.075	0.626	0.058	0.056	0.049	0.061	0.051	0.052	0.694	0.617

Only 7 animals tested