DISSERTATION

Isolation and characterization of immunoglobulin G from *Panthera leo* in South Africa and Zimbabwe

By

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DEDICATION

This dissertation is dedicated to my loving five-year old daughter, Lehlogonolo Manamela who has been there for me throughout this process. Your smile, laughter and love have given me the courage to complete this dissertation. My late mother, Mrs Grace Manamela and sister, the late Eunice Manamela, I owe you my achievement because you believed in me and raised me well. I am grateful for your strictness, love and support.

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ABSTRACT

While a decrease of wild felid population has led to disruption of conservation programme, recent studies have shown the importance of immune regulation for determining health outcomes and co-infection. Immunoglobulin G is important for detecting and evaluating responses to infectious diseases and vaccination. But, there is limited information on felid immunoglobulins and their role for functional immunity.

This study aimed at isolating and characterizing lion's immunoglobulin G. Lions' sera (*n* = 68) were processed using the MagReSyn® magnetic beads and the final protein concentration was determined using the Xpose™ Trinean Spectrophotometer. The cross-reactivity of goat anti-cat immunoglobulin with sera of lions and other species was analysed using ELISA. High cross-reactivity was observed in lions ranging from 87.7 to 100%, and low reactivity with rhino (22.4%) followed by chicken (0.01%). The protein concentration from purified sera yielded 39.09 mg/ml. Molecular weight of lion IgG 150-160 kDa was detected with both chains at 54-56 kDa and 24-26 kDa on SDS-PAGE. These results indicate a potential aid in developing serological tools to monitor exposure to micro-organisms of lions.

Key words*: *African lions, Panthera leo, Immunoglobulin G, ELISA, SDS-PAGE, Purification, Protein concentration, Cross-reactivity, Molecular weight

LIST OF ABBREVIATIONS

%	Percentage
γ	Gamma
°C	Degree Celsius
µg	Microgram
µg/ml	Microgram per millilitre
µl	Microlitre
µl/mg	Microlitre per milligram
µl/ml	Microlitre per millilitre
APS	Ammonium persulfate
BCRs	B-cell receptors
CD4+	Cluster of differentiation 4
CD8+	Cluster of differentiation 8
CDV	Canine distemper virus
C_L	Constant light region
CMI	Cell-mediated immunity
CoViD-19	Corona-virus-disease 2019
DAFF	Department of Agriculture, Forestry and Fisheries
DNA	Deoxyribonucleic acid
DVTD	Department of Veterinary Tropical Diseases
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen- binding
Fc	Fragment crystallizable region
FCoV	Feline coronavirus
FCV	Feline calicivirus
FECV	Feline enteric coronavirus

FHV	Feline herpes virus
FIPV	Feline infectious peritonitis virus
FIV	Feline immunodeficiency virus
FPLV	Feline panleukopenia virus
FV	Final value
H	Heavy chain
H_2SO_4	Sulphuric acid
HCT	Haematocrit
HGB	Haemoglobin
HI	Humoral immunity
HRP	Horseradish peroxidase
IFA	Indirect fluorescent antibody
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Igs	Immunoglobulins
IHA	Indirect haemagglutination assay
k	Kappa
kDa	Kilo Daltons
km^2	Square kilometre
KNP	Kruger National Park
L	Light chain
M	Molar
MCH	Mean corpuscular haemoglobin

MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
Mg	Milligram
mg/ml	Milligram per millilitre
Ml	Millilitre
NaCl	Sodium chloride
nM	Nanomolar
OD	Optical density
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
pH	Potential of Hydrogen
PLT	Platelet
PVs	Papillomaviruses
RBC	Red blood cells
RNA	Ribonucleic acid
Rpm	Revolutions per minute
SARS-CoV-2	Severe acute respiratory syndrome coronavirus-2
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium-dodecyl sulfate polyacrylamide gel electrophoresis
sp.	Species
TEMED	Tetramethylethylenediamine
TMB	3,3′,5,5′-Tetramethylbenzidine
UNISA-CAES	University of |South Africa- College of Agriculture and Environmental Sciences
V_H	Heavy chain variable domain
V_L	Light chain variable domain

WBC	White blood cells
A	alpha
Δ	Delta
E	Epsilon
Λ	Lambda
M	Mu

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Chapter 1: Literature Review

Increasing anthropogenic pressure on economically and ecologically important domestic and wildlife species, as well as the recognition of certain species as a viable model for both human and animal diseases, has led to the expansion of animal health research worldwide (Broughton, 2017, Hassell et al., 2017). As these changes occurred, domestic species, including cats (*Felis catus)* have been used to study immune mechanisms for disease susceptibility, because of their social importance to humans as members of the family, as well as the analogous nature of their immune system to that of humans (Combes, 1996, Pastoret et al., 1998). However, while a large body of scientific evidence now exists to characterize most branches of the immune system in domestic cats, less is known about immune profiles of free-ranging felids, including lions (*Panthera leo*) due to the general inaccessibility of samples from these species.

Populations of wild felids are vulnerable and decreasing in their natural habitat. This is due to biosphere changes, prey depletion, human-lion conflict and exposure to infectious diseases of domestic animals, humans, and other wildlife species, which act as continual reservoirs of infection (Kelly et al., 1993, Thalwitzer et al., 2010, Brown, 2011, Packer et al., 2011, Riggio et al., 2013, Henschel et al., 2014, McDermid et al., 2017). In view of the above mentioned threats, understanding the immune mechanisms that may underlie disease susceptibility and provide protection against large scale population crashes in the face of disease outbreaks has become paramount (Dalerum et al., 2008, Ferreira et al., 2013). Hyenas like other predators that hunt and scavenge namely black backed jackal, have shown the ability to survive infectious diseases such as anthrax, rabies and other infectious diseases of carnivores (Bellan et al., 2012, Flies et al., 2012).

The Kruger National park (KNP) is one of the largest game reserves in Africa and covers an area of 19 485 km^2 (Joubert, 1986). This area is home to a diverse population of wild animals, which include small felids such as caracal (*Caracal caracal*), servals (*Leptailurus serval*) and African wild cats (*Felis lybica*) and large felids such as cheetahs (*Acinonyx jubatus*), leopards (*Panthera pardus*) and African lions (*Panthera leo*) (Fairall, 1968).

Cheetah and leopard populations in the park are decreasing due to their susceptibility to infectious diseases such as tuberculosis (Thalwitzer et al., 2010, Viljoen et al., 2015).

Lions have a relatively large population in the park compared to other felids, bolstered mostly by a higher rate of survival due to their affiliation with social groups (prides) consisting of more than one male, which provides protection for young and territory, as well as increased efficiency with regards to prey handling (Dalerum et al., 2008, Viljoen et al., 2015). In addition, they feed on a variety of prey species and have low water requirements due to their ability to meet hydration needs through consumption of water in prey tissues, conferring resistance against droughts in the face of large die-offs of other species (Eloff, 1973, Funston et al., 1998). Despite this resilience, recent years have seen a decrease in lion populations due to increasing pressure from poachers along park boundaries, as well as local epizootics caused by diseases transmitted by sympatric species (Ferreira et al., 2013).

1.1 Infectious diseases of lions

Past studies investigating bacterial, viral, protozoal and other parasitic diseases in lions and other felid populations have shown KNP to have a high disease prevalence throughout various regions of the park (Antunes et al., 2008, Ferreira and Funston, 2010, Maas et al., 2010, Maas et al., 2012b, Broughton, 2017). Furthermore, recent studies have shown the importance of immune regulation for determining health outcomes and co-infection dynamics as regulated by viral and parasitic coinfections of known importance (Broughton, 2017). Despite these findings, there is limited information on felid immunoglobulins and their role for functional immunity in these threatened species.

1.1.1 Bacterial diseases of lions

Some bacteria species in the genera of *Bartonella, Mycobacterium* and *Mycoplasma* have been reported to cause disease in domestic cats and free-ranging lions (Viljoen et al., 2015, Molia et al., 2016).

Bartonellaceae are aerobic, gram-negative bacteria transmitted by vectors such as lice, flea, sandflies and ticks (Noguchi, 1926, Jacomo et al., 2002, Billeter et al., 2008, Chomel et al., 2009). The bacteria infect erythrocytes, endothelial cells and macrophages which leads to disease (Billeter et al., 2008). *Bartonellae* species such

as *Bartonella clarridgeiae* and *Bartonella koehlerae* have been isolated from animals and humans, and felids are found to be a source of infection (Molia et al., 2004). Cat scratch disease is the most common disease in domestic cats and other felids, including lions caused by *Bartonella henselae (B. henselae) (Pretorius et al., 2004, Chomel et al., 2006, Molia et al., 2016)*. Clinical symptoms of *Bartonella* species include fever (in humans), swollen lymph nodes, endocarditis and neuroretinitis (Rotstein et al., 2000, Molia et al., 2004, Pretorius et al., 2004). In the study conducted by Molia et al. (2004), *B. henselae* was identified in both lions and cheetah by polymerase chain reaction (PCR) using cultured colonies from whole blood pellets. *Bartonella henselae* was also isolated by culture and identified using PCR and ELISA in lions from three ranches in the Free State Province (Pretorius et al., 2004).

Mycobacterium bovis is a gram-positive rod-shaped bacterium, non-motile, aerobic, does not form any spores and is acid fast staining (Quinn et al., 1994). *M. bovis* organisms are slow growers and culture results can take up to 16 weeks to be confirmed as negative (Wadhwa and Mahajan, 2006). *Mycobacterium bovis, a* member of the *Mycobacterium tuberculosis* complex (MTBC), which also includes *M. tuberculosis*, *M. caprae*, *M. microti*, *M. africanum*, *M. canettii* , *M. pinnipedii, M. orygis*, *M. bovis,* bacilli Calmette-Guérin (BCG), *M. mungi*, *M. suricattae* and the dassie bacillus (Bass et al., 2013). *Mycobacterium bovis* causes tuberculosis in domestic and wild animals (Michel et al., 2010, Musoke et al., 2015).

There have been reports on African wildlife species infected with *M. bovis* since 1929. These include primates and non-primates (Keet et al., 1996, De Vos et al., 2001, Cleaveland et al., 2005, Michel et al., 2006, Trinkel et al., 2011, Viljoen et al., 2015). Tuberculosis was first detected in the 90's from African buffalo in the KNP and since then has spread to other parts of the park to a diverse number of wildlife species including lions (Michel et al., 2006, Viljoen et al., 2015). The route of exposure to other species including lions has been studied in detail and Miller et al. (2015) confirmed infection through the respiratory system using tracheobronchial lavage samples from the KNP lions.

M bovis in lions was first reported in the KNP in 1996 (Keet et al., 1996, Sylvester et al., 2017). Lions can contract an *M. bovis* infection by consuming infected prey, such as buffalo, the reservoir host of the disease in South Africa (Keet et al., 1996, Viljoen

et al., 2015). The main clinical symptom caused by *M bovis* in lions is emaciation and pulmonary and bone lesions post-mortem (Keet et al., 1996, Miller et al., 2019). The diagnosis of *M bovis* infection in lions has been documented and included post mortem examination, isolation on culture, microscopic examination and histopathology examination, immunological assays (ELISA, interferon-gamma assay and tuberculin skin test) and molecular tests (PCR) (Maas, 2011, Viljoen et al., 2015, Miller et al., 2019, Viljoen et al., 2019).

Bacillus anthracis is an aerobic or facultative anaerobic gram-positive bacterium that forms spores (Russell et al., 2008, Koehler, 2009). The bacteria is extracellular with an intracellular presence during pulmonary anthrax (Russell et al., 2008). The ubiquitous bacterium can survive for a long time in dried culture and can remain viable in soil for many years (Smith et al., 2000, Lembo et al., 2011, Steenkamp et al., 2018). Anthrax is a contagious disease of domestic, wild mammals and humans (Hugh-Jones and De Vos, 2002, Hampson et al., 2011). Anthrax in lions in KNP has been diagnosed and infection is through opening of carcass by scavengers, feeding from infected prey and drinking from infected water holes (Hugh-Jones and De Vos, 2002). The swelling of the head is one of the clinical signs observed in the early stages of infection in lions. Lions develop a strong (antibody) immunity to anthrax after the first few exposures. Histopathology, serologic testing (ELISA), bacterial culture and molecular techniques are used for identification of the bacteria (WHO, 2008, Range, 2011).

Clostridium is a gram-positive spore-forming anaerobic bacteria causing diseases in humans and animals (Rorbye et al., 2000, Greco et al., 2005). The natural habitat of *Clostridium* species are the soil, water and gastrointestinal tract of both animals and humans (Haagsma, 1991, Rorbye et al., 2000, De la Fe et al., 2006). *C.tetani, C. perfrigens, C. botulinum, C. septicum and C. bifermentans* are *Clostridium* species isolated from humans and animals (De la Fe et al., 2006). De la Fe et al. (2006) reported the first case of *C. sordellii* in lions. The clinical symptoms observed include ataxia, paralysis and exhaustion (Greenwood, 1985, De la Fe et al., 2006). Cases of *C. perfringens* and *C. sordellii* were also identified and isolated in other felines (Greco et al., 2005, Zhang et al., 2012). Polymerase chain reaction test, bacteriological culture, gram-stain technique and indirect fluorescent antibody (IFA)

test are often used to identify the bacteria (Greco et al., 2005, De la Fe et al., 2006, Zhang et al., 2012).

1.1.2 Viral diseases of lions

Diseases of wild carnivores have been studied worldwide with outcomes of most carnivores surviving other diseases (Flies et al., 2012). Wild felids are susceptible to many viruses (canine distemper virus, feline immunodeficiency virus, feline leukaemia virus, feline panleukopenia virus) affecting domestic cats and humans (Spencer, 1991, Endo et al., 2004, Dybas, 2009).

Feline panleukopenia virus (FPLV) belongs in the Parvoviridae family. FPLV is a single-stranded DNA virus of about 18 to 26 nm in diameter (Povey and Davis, 1977, Agbandje et al., 1993). Feline panleukopenia has been reported in lions and other species with the first report diagnosed from a leopard (Johnson, 1964, Povey and Davis, 1977, Spencer, 1991, Endo et al., 2004). Spencer (1991) then confirmed the occurrence of FPLV in free-ranging lions in the KNP.

Canine distemper virus (CDV) is a member in the family of *Paramyxoviridae*, genus *morbillivirus*. This enveloped single-stranded, negative sense RNA virus has viral attachment spikes (Bellini et al., 1986, De Vries et al., 2015, Rendon-Marin et al., 2019). As an infectious disease, canine distemper is recognized worldwide and has been reported in carnivores (Deem et al., 2000, Loots et al., 2018). Canine distemper disease was diagnosed in lions from the Serengeti National Park in Tanzania in the 90's using histopathology and serology test (Roelke-Parker et al., 1996). This outbreak in lions and other large felids resulted in the death and decline in the lion population (Roelke-Parker et al., 1996, Endo et al., 2004). In addition, large felines that are tested serologically for FPLV and CDV also have antibodies against feline immunodeficiency virus (FIV). The felids have extensive exposure to most of the common feline and canine viruses (Roelke-Parker et al., 1996, Endo et al., 2004, Driciru et al., 2006).

Gaskell et al. (2007) describe feline herpes virus (FHV) as an alpha herpesvirus of cats closely related to canine herpesvirus-1 and porcine herpesvirus-1. Hofmann-Lehmann et al. 1996 confirmed the prevalence of FHV in lions using Enzyme-linked

immunosorbent assay (ELISA). Feline herpes virus and other viruses (FIV, FCV, feline parvovirus, feline coronavirus and CDV) of felids were also diagnosed in lions of Serengeti and Tanzania in 1999. Feline leukaemia virus was not detected from the lions in the studies conducted using serological tests (Hofmann-Lehmann et al., 1996, Packer et al., 1999).

Feline calicivirus (FCV) is a small single-stranded, positive-sense RNA virus of cats and is widespread in the feline population (Maeda et al., 1998, Radford et al., 2007, Thiry et al., 2009, Henzel et al., 2015). The pathogen has been reported in African lions (Martella et al., 2007). Nasal, oral or conjunctival are routes of infection (Radford et al., 2007). Together with FHV, the pathogen is identified from cellular culture, IFA, serology and PCR (Henzel et al., 2015). The immune response against FCV infection depends on host factor and feline immune status (Radford et al., 2007, Henzel et al., 2015).

Feline immunodeficiency virus is a T-lymphotropic pathogenic lentivirus of domestic cats and has been detected in several non-domestic feline species (Callanan et al., 1992, VandeWoude et al., 1997, Roelke et al., 2006). The FIV causes immunosuppression in domestic cats (Brown et al., 1994, Roelke et al., 2009). It was discovered by (Pedersen et al., 1987) as the etiologic agent of an immunodeficiency syndrome in cats (Roelke et al., 2006). Studies have shown that FIV can infect lions (Brown et al., 1993, Brown et al., 1994, Poli et al., 1995, Hofmann-Lehmann et al., 1996, Troyer et al., 2005, Brennan et al., 2006, Roelke et al., 2006, Maas et al., 2012a). Free-ranging lions carry the FIV (lion) (FIV-Ple), which is the chronic species-specific strain of FIV (Troyer et al., 2005, Roelke et al., 2009). In most studies, serological tests (ELISA, IFA, Western Blot techniques) are conducted to detect the antibodies to FIV (Hofmann-Lehmann et al., 1996). Molecular cloning and complete nucleotide sequencing are also used (Olmsted et al., 1989, Poli et al., 1995, McEwan et al., 2008). Immune (CD4+ T-lymphocytes) depletion is recorded as one of the pathological conditions related to lentivirus infections (Roelke et al., 2009).

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) which causes corona-virus-disease (CoViD-19) in humans was also detected in some animals (Newman et al., 2020, Sit et al., 2020). It is a single-stranded positive sense RNA virus with spike proteins (Newman et al., 2020, Sit et al., 2020) and appears spherical

in shape (Tufan et al., 2020). The Wildlife Conservation Society's Bronx Zoo in New York reported the first natural infection case of SARS-CoV-2 cases in lions and tigers. Zoo keepers, who tested positive for the infection, have transmitted the virus to the animals since they had close contact with the animals (McAloose et al., 2020). African lions are also reported to be infected with feline coronavirus (FCoV), a contagious pathogen of domestic and non-domestic Felidae (Kennedy et al., 2002, Kennedy et al., 2003, Stephenson et al., 2013). The two forms of FCoV include feline enteric coronavirus (FECV) which causes intestinal infection in young felids (Pedersen et al., 1981) and feline infectious peritonitis virus (FIPV) causing feline infectious peritonitis (FIP), a fatal immune-mediated vasculitis of felids (Poland et al., 1996, Kennedy et al., 2003, Stephenson et al., 2013). Clinical symptoms associated with FCov infection are fever, weight loss and chronic diarrhoea (Kennedy et al., 2002, Stephenson et al., 2013). PCR and IFA are mostly used to identify the virus (Kennedy et al., 2002, Kennedy et al., 2003, Stephenson et al., 2013).

Papillomaviruses (PVs) are small, non-enveloped, double-stranded DNA viruses. They are highly species specific pathogens of felids (Sundberg et al., 2000, Rector et al., 2007). All PVs belong to the Lambda papillomavirus genus (Rector et al., 2007). The infected species develop localised proliferative lesions caused by injection of the infectious virions through the skin surface. Papillomaviruses are also thought to cause feline sarcoids, a cutaneous fibropapilloma (Carney et al., 1990, Hanna and Dunn, 2003). This virus was identified in Persian cats using electron microscopy technique and immunohistochemical analysis (Carney et al., 1990). In 2000, (Sundberg et al.) reported feline papillomavirus in six cat species (*Felis domesticus, Felis concolor, Felis rufus, Panthera leo, Panthera uncia* and *Neofelis nebulosa*) using histological and immunohistochemistry techniques. The cat lesions had similar clinical appearance to those in humans.

1.1.3 Protozoal diseases of lions

Protozoan parasites, such as *Leishmania*, *Giardia*, *Cryptosporidium* and *Toxoplasma* parasites are found in felids. These parasites can be transmitted via consumption of either undercooked food or contaminated water and arthropod vectors (Bjork et al., 2000, Otranto et al., 2015).

Toxoplasma gondii is a coccidian parasite of felids and humans and is divided into three stages, the tachyzoites, bradyzoites and sporozoites. In the tachyzoites stage, the parasites enter the host cell and multiply rapidly. Bradyzoites include the slow multiplication of cysts within the tissue and sporozoites stage occur in the oocysts and sporulation occurs (Dubey et al., 1998, Tenter et al., 2000). Felids are the only known definitive hosts of *Toxoplasma gondii* shedding oocysts in the environment and providing the infection to other warm-blooded animal species (Lappin et al., 1991, Yang et al., 2017, Ferra et al., 2020). Riemann et al. (1975) reported the first case of *Toxoplasma gondii* in a lion from Serengeti National Park, Tanzania. The lion was seropositive to *Toxoplasma gondii* using indirect haemagglutination assay (IHA) (Penzhorn et al., 2002). *Toxoplasma gondii* and *Neospora caninum* are closely related parasites of felines and canids (Kamga-Waladjo, 2009, Pedrosa, 2018). The two parasites were investigated in lions using serum samples for ELISA to examine antibodies against Toxoplasma *gondii* and *Neospora caninum,* respectively. The serological results indicated more exposure to *Neospora caninum* than *Toxoplasma gondii*. Agglutination test and PCR are also used to detect the presence of *Toxoplasma gondii (Kamga-Waladjo, 2009)*. Lions, like other felids are the only definitive hosts of *Toxoplasma gondii.* The oocysts shed in the faeces provide an infection in various warm-blooded animal species (Yang et al., 2017). The infection of protozoan parasite remains prevalent in lions and other wild felids (Ferreira et al., 2019, Seltmann et al., 2020).

Cryptosporidium and Giardia are parasites that can infect domestic animals, wild animals and humans causing diarrhoea and other enteric disorders. The transmission of these parasites is by pollution from faecal material of both humans and animals. Molecular tests have shown that both strains of *Cryptosporidium and Giardia* are found in captive and free-ranging wildlife animals (Appelbee et al., 2005).

Babesia are tick-transmitted hemoprotozoans that infect mammals and birds (Schnittger et al., 2012). Penzhorn et al. (2001) reported the first characterization of felid babesia parasite in lions using PCR. Various wild carnivores have been reported to have *babesia*. *Babesia* species have also been described from different animal species namely dogs, mongoose, hyena, racoons, rhinoceros, elephants, Bovidae, Cervidae and felids. All the lions in the KNP tested during the study were found to be

infected with *Babesia leo* (Penzhorn, 2006). *Babesia* was also reported in the lions of the Serengeti in Tanzania as a threat to the future of lions (Dybas, 2009).

Other vector borne diseases are transmitted by Ixodid ticks, ectoparasites also found in wild felids. *Amblyoma sp*, *Hyalomma sp*. and *Rhipicephalus sp*. were identified in wild felids, including lions. In the study by Horak et al. (2010) cats and wild felids were concluded as good hosts for *Hyalomma elliptica* (Horak et al., 2010).

1.1.4 Other parasites of lions

Other parasites in African lions have been reported across Africa (Bjork et al., 2000, Hüttner et al., 2008). The table below summarises the studies on micro and macro parasites of African lions. Bjork et al. (2000) identified 19 different parasites from the free-ranging African lions in the Serengeti national park and Ngorongoro conservation area. Freshly defecated faecal samples were collected. The study indicated that the structures of cestode and trematode studied could have originated from other animal species and do not all represent the true parasite of lions.

Table 1: Summary of parasites identified in African lions

Parasite	Place	Samples	Tests conducted
Cestoda			
Spirometra sp.	Serengeti and Ngorongoro Crater, Tanzania	Faeces	Egg counts
Echinococcus felidis	Uganda	Faeces	Egg counts and nuclear DNA sequencing
Taenia sp.	Tanzania	Faeces	Egg counts
Anoplocephalidae	Serengeti and Ngorongoro Crater, Tanzania	Faeces	Egg counts
Trematoda			
Trichuris sp.	Serengeti and Ngorongoro Crater, Tanzania	Faeces	Egg counts
Nematoda			
Dirofilaria	Aitana Safari Park, Spain	Tissue (Lung)	Histological technique
Aelurostrongylus sp	Serengeti and Ngorongoro Crater, Tanzania	Faeces	Egg counts
Trichinella sp.	Greater Kruger National Park, South Africa	various	Various
Capillaria sp.	Serengeti and Ngorongoro Crater, Tanzania	Faeces	Egg counts
Habronema sp.	Serengeti and Ngorongoro Crater, Tanzania	Faeces	Egg counts
Others			
Acanthocephala	Serengeti and Ngorongoro Crater, Tanzania	Faeces	Egg counts
Demodex sp.	Serengeti and Ngorongoro Crater, Tanzania	Faeces	Egg counts
Eimeria sp.	Serengeti and Ngorongoro Crater, Tanzania	Faeces	Egg counts
Isospora sp.	Serengeti and Ngorongoro Crater, Tanzania	Faeces	Egg counts

(Müller-Graf et al., 1999, Bjork et al., 2000, De Ybanez et al., 2006, Hüttner et al., 2008)

1.2 General immunology

Recent studies have shown the importance of immune regulation for determining health outcomes and coinfection dynamics as regulated by viral and parasitic coinfections of known importance (Broughton, 2017). Despite these findings, there is limited information on felid immunoglobulins and their role for functional immunity in these threatened species.

Delves and Roitt (2000) describe the immune system as a collection of cells and molecules that help protect against infection (Parham, 2014). It has two major arms, innate immunity which is present from birth and adaptive immunity which develops from birth and continues to do so as the individual is exposed to different antigens during its lifetime (Roitt et al., 1989, Beutler, 2004, Turvey and Broide, 2010). Microbial infections are recognised by the innate immune system to produce immediate defence and to also generate a long-lasting adaptive immunity (Iwasaki and Medzhitov, 2015).

1.2.1 Innate immunity

Innate immunity represents the principal defence machinery in vertebrates. It responds in minutes and hours of an antigen appearance to the body. It is non-specific and does not exhibit memory. It is the first line of defence that fights any foreign invaders and is found in organisms such as insects and plants (Roitt et al., 1989, Tizard Ian, 1996, Bonilla and Oettgen, 2010).

1.2.2 Adaptive immunity

Adaptive immunity also known as specific immunity is a lymphocyte dependent immune system with various antigen receptors and exhibits immunologic memory. Adaptive immunity consists of cell-mediated immunity (CMI) and humoral immunity (HI). Cell-mediated immunity is mediated by T-cells and targets intracellular-pathogens. In CMI, the effector phase is initiated by T-cells recognizing the peptide-Major Histocompatibility Complex (MHC) antigens on activated antigen presenting cells. These cells play a role in eliminating microbes and other sources of antigen by, stimulating inflammation through secretion of cytokines and killing pathogen infected cells and phagocytosed and extracellular microbes. There are two response types of CMI, the CD4+ helper T-cell which respond to microbes phagocytosed by phagocytes and the CD8+ cytolytic T-cell which responds to microbes that infect and replicate

intracellularly in various cell types (Roitt et al., 1989, Bonilla and Oettgen, 2010, Tubo and Jenkins, 2014).

Humoral immunity is mediated by antibodies and targets extra-cellular pathogens. During HI, antibodies are secreted by activated B-lymphocytes (plasma cells) and bind to an antigen. The antigen will then be neutralized, and phagocytosis will take place. Adaptive or active immunity can be stimulated by an infectious agent or by immunization/ vaccination. This is in contrast to passive immunity conferred by antibodies present in body fluids, which can be transferred to another individual to provide protection (Roitt et al., 1989, Bonilla and Oettgen, 2010).

1.2.3 Passive and active immunity

Immunity is the state of resistance to an infection and has both specific and non-specific mechanisms. The state of resistance to an infection can be acquired by passive or active immunity (Tizard Ian, 1996).

1.2.3.1 Passive immunity

During passive immunization, antibodies are transferred from a resistant animal to a susceptible animal for immediate protection against an antigen such as, antibody transfer naturally from mother to baby via placenta and from breast milk/ colostrum or artificially from an immune donor. It can also be induced artificially where antibodies produced in a donor animal by active immunisation are administered to susceptible animals for immediate immune protection (Roitt et al., 1989, Tizard Ian, 1996).

1.2.3.2 Active immunity

Active immunity is induced after the administration of antigen to an animal to provide a long lasting protective immune response. There are two types of active immunity, natural and artificial immunity. Natural immunity is activated after exposure to the antigen and happens naturally. Artificial immunity is induced after a vaccine is administered and the animal develops its own antibodies. Vaccinations are made of specific antigens; they are administered to protect animals against infectious diseases and generate an active immune response. Exposure of disease results in cells of the animal's immune system interacting with the organism, therefore antigen producing cells must be stimulated followed by stimulation of B-cells and T-cells to produce large number of memory cells (Roitt et al., 1989, Tizard Ian, 1996).

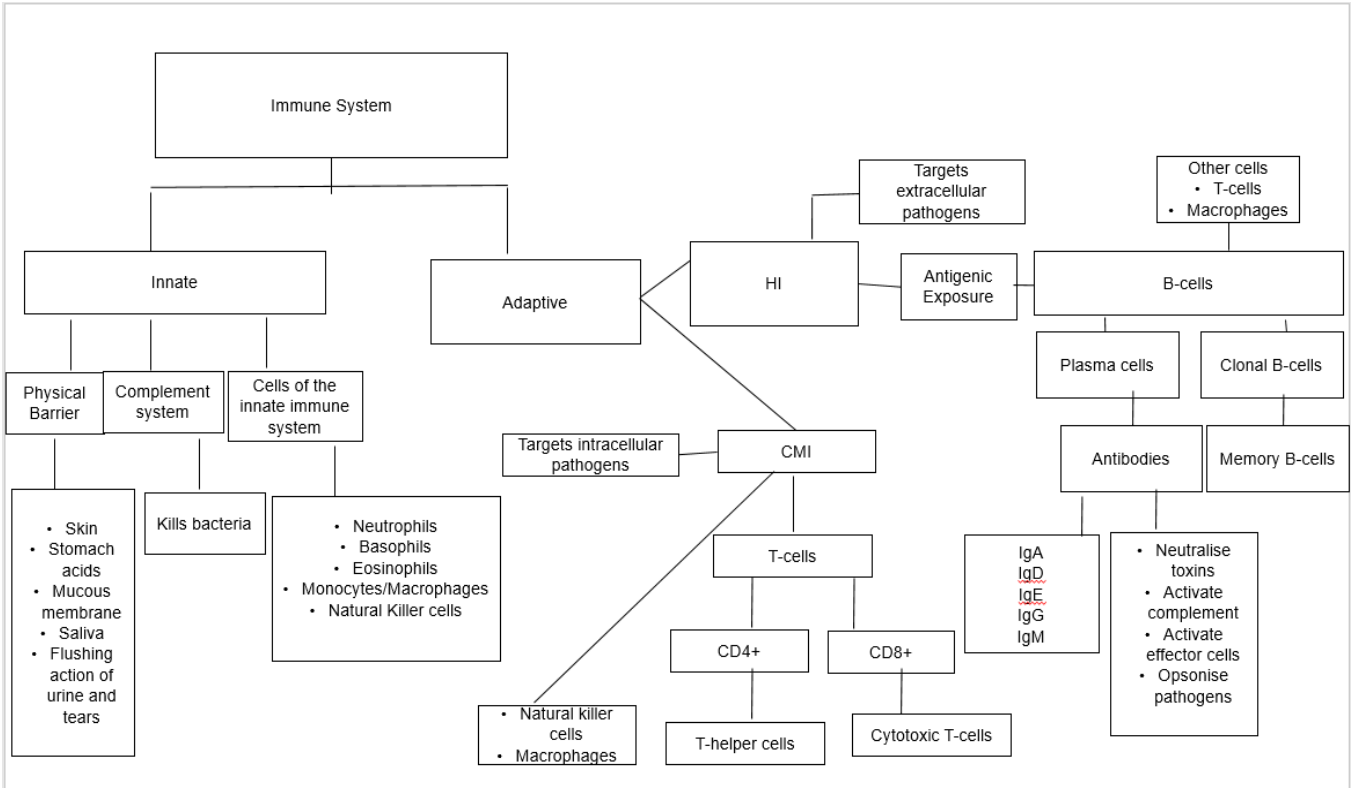

Figure 1: A brief overview of the immune system and functions (Roitt et al., 1989, Tizard Ian, 1996, Tizard, 2013).

1.3 Immunoglobulins

The immunoglobulins (Igs), also called antibodies, are a group of glycoproteins found in serum, plasma and tissue fluids of all mammals. The major proteins found in blood are fibrinogen (only in plasma), globulins and albumins. The maturity of the B-cells takes place in the bone marrow and then develop into lymphocytes responsible for antibody production and plasma cells in response to an antigen. B-cells express proteins (immunoglobulins) on their cell surfaces. The B-cell receptors (BCRs) are formed by immunoglobulins and secrete the same immunoglobulins circulating as antibodies. These immunoglobulins bind to a specific antigen (Gally, 1973, Nisonoff, 1983, Tizard Ian, 1996, Frank, 2002, Bhattacharya, 2008).

The basic structure of an Ig consists of disulphide bonds and four polypeptide chains, the two light chains (L) and the two heavy chains (H) containing variable regions (V_H or V_L) at the N terminal. The C terminal is part of the constant region. The light chains have a molecular mass of 25 000 Daltons and the heavy chains have 70 000 Daltons. The light chain and heavy chain are linked by a disulphide bond to form an H-L bond. The two H-L bonds are then connected by another disulphide bond to form a full

structure of immunoglobulin (Neoh et al., 1973, Huse et al., 2002, Bhattacharya, 2008, Schroeder Jr and Cavacini, 2010).

The IgG molecule as shown in figure 2 consists of two identical fragments, antigen binding (Fab) region; the antigen binding site and one Fc (fragment, crystallisable) region both connected by a hinge region which allows the distance between the two antigen-binding sites to vary and is found only in IgG, IgA and IgD. IgM and IgE lack the hinge region, instead each have an additional constant domain. Each of the Fab regions consists of two domains, the V_H-V_L and C_H1-C_1 whereas the Fc region has two or three domains (Tizard Ian, 1996, Bhattacharya, 2008).

The amino acid sequence found in the light chain on the N-terminal are different hence called the variable light region (V_L). Half of the amino acid sequence on the C-terminal of each light chain are identical and called the constant light region (C_L) with two types the kappa ($_k$) and the lambda (λ) light chains (Tizard Ian, 1996, Bhattacharya, 2008). Bhattacharya (2008) reported that, in humans, the heavy chain of the IgG consists of about 445 amino acids with 115 at the N-terminal and the remaining 330 at the constant heavy chain region. This sequence corresponds to the five different heavy chains, alpha (α), epsilon (ε), mu (μ), delta (δ) and gamma heavy chains (γ) to define the classes of the immunoglobulins (Tizard Ian, 1996, Bhattacharya, 2008).

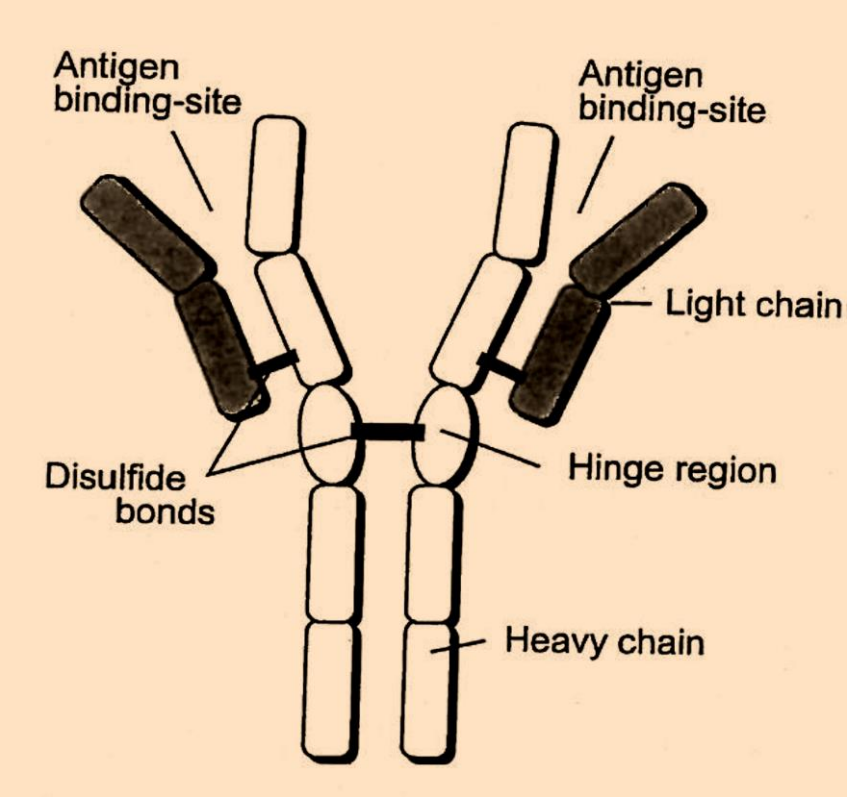

Figure 2: Structure of an immunoglobulin molecule (Tizard Ian, 1996).

There are five major classes of heterodimeric proteins called immunoglobulins (IgA, IgD, IgE, IgG and IgM) (Tizard Ian, 1996, Huse et al., 2002). Immunoglobulins are responsible for humoral immunity, which is a branch of the adaptive (specific) immune system used largely for adaptive immune memory; opsonisation (identification) of foreign pathogenic and parasitic invaders; and neutralization of small invading pathogens and toxins (Tizard Ian, 1996, Sommer, 2005, Tizard, 2013, Moticka, 2015, Tao and Xu, 2016). In general, immunoglobulins can be found in serum, on the surface of cells, and in secretory fluids such as colostrum, bronchial exudates, saliva, and nasal secretions (Lieberman, 2002). In cats and dogs, IgG and IgA are found in high concentration in colostrum, which is the "first mammary secretion rich in antibodies" that the kittens and or puppies receive after birth. IgG is found in higher concentrations in feline milk whereas IgA is found in higher concentrations in canine milk (Heddle and Rowley, 1975, Casal et al., 1996, Day, 2007). In 1973, (Neoh et al.) studied the immunoglobulins of humans, artiodactyls, perissodactyla, proboscidea, pinnipedia, lagomorpha, rodentia, marsupalia, and carnivores using chicken antisera. Findings of that study showed evidence of high cross-reactivity between species, with

every animal tested, including lions and cheetahs, showing profiles predominated by IgG (Vaerman et al., 1969, Neoh et al., 1973). Anti-cat isotype-specific antibodies are commercially available to assess cross-reactivity in lions, as they are evolutionarily related to cats (Flies et al., 2012).

1.3.1 Immunoglobulin A

The discovery of immunoglobulin A (IgA) based on mucosal immunity was introduced by Joe Heremans in 1959 (Tomasi, 1992, McDermid et al., 2017). The individual IgA molecules have a molecular weight of 150 kDa (Tizard Ian, 1996). Immunoglobulin A is secreted by plasma cells on the mucosal surfaces of the eyes, mammary glands, respiratory tract, skin and urogenital areas for protection against invading microorganisms. It consists of about 10% to 15% of the total serum concentration and normally secreted in a dimer form (Tizard Ian, 1996, Bhattacharya, 2008).

1.3.2 Immunoglobulin D

The immunoglobulin study from myeloma patients introduced another type of Ig, immunoglobulin D (IgD) by David Rowe and John Fahey (Rowe et al., 1973, Preud'homme et al., 2000). Immunoglobulin D is detected in low concentrations in plasma, less than 1% of the total serum concentration is found but not in all species. Immunoglobulin D together with IgM are expressed by mature B-cells. Immunoglobulin D molecule consists of two delta heavy chains and two light chains, kappa or lambda chains lacking inter-chain disulphide bonds with a molecular weight of about 170 kDa (Tizard Ian, 1996, Bhattacharya, 2008).

1.3.3 Immunoglobulin E

Immunoglobulin E (IgE) was discovered by Kimishige and Teruko Ishizaka in the 1960s (Ribatti, 2016). The study was based on antibody involving allergic reaction releasing histamine and this has led to the findings on treatment for patients with allergy and improvement on allergy diagnosis (Johansson, 2011, Ribatti, 2016). Immunoglobulin E is found in low concentrations in serum with a molecular weight of 190 kDa (Tizard Ian, 1996). Immunoglobulin E is made by plasma cells on the surface of mast cells in the tissues. It is found in low concentrations in serum and can only act as a signal-transducing molecule. The Fc region binds strongly to receptors on mast cells and basophils releasing inflammatory agents such as histamine to eliminate the pathogen. Immunoglobulin E has the shortest half-life of all the immunoglobulins (2

to 3 days) and a mild heat treatment can destroy it easily (Tizard Ian, 1996, Bhattacharya, 2008).

1.3.4 Immunoglobulin G

Immunoglobulin G (IgG) shown in figure 2 is the most common immunoglobulin with the highest concentration in serum and plays a major role in the antibody-mediated defence mechanisms as a type of passive immunity (Tizard Ian, 1996, Bhattacharya, 2008). The structure of IgG (Fc and Fab fragments) was discovered by Rodney Porter using the enzyme papain (Porter, 1973). Gerald Edelman then discovered the four chains (heavy and light chains) of immunoglobulins (Porter, 1973, Raju, 1999). Immunoglobulin G can also promote phagocytosis by binding to a molecule using Fc receptors (opsonisation) (Nezlin, 2017). It can agglutinate, precipitate antigen and activate the pathway of complement when there are sufficient molecules accumulated. It has a molecular weight of about 180 kDa and can easily escape from blood vessels (Tizard Ian, 1996, Bhattacharya, 2008).

1.3.5 Immunoglobulin M

Immunoglobulin M (IgM) is the first immunoglobulin produced among other immunoglobulins. The main function of IgM has the ability to bind to multiple foreign antigens (Zhou et al., 2007, Ehrenstein and Notley, 2010, Dimitrov et al., 2013) . Immunoglobulin M is secreted by plasma cells and found in the second highest concentration after the IgG in serum. It constitutes of about 5% to 10% of the total serum concentration. IgM is made of five monomeric units linked by disulphide bonds with a molecular weight of 900 kDa. It is produced in the primary response to an antigen and considered as the strongest complement activator, for opsonisation, neutralization of viruses and for agglutination due to its large size (Tizard Ian, 1996, Bhattacharya, 2008).

1.4 Development of monoclonal antibodies

According to Capers (2006), one can use the immunoglobulin purification techniques that have been used previously for purifying the immunoglobulin of other species to study the immune system of different unstudied species. Commercial antisera developed against the immunoglobulins of humans, mice, dogs and sheep are available. These species' immunoglobulins have been studied in detail and can limit the time in developing new antisera (Capers, 2006).

(Cavagnolo and Vedros, 1978, Azwai et al., 1993, Kelly et al., 1998) in their studies performed an immunosorbent assay for cross-reactivity using different monoclonal and polyclonal antibodies produced against different immunoglobulin classes and subclasses of different animals. There was no cross-reaction between the monoclonal antibodies and the immunoglobulin of African elephant (*Loxodonta africana*) and camel (*Camelus dromedarius*). The polyclonal antibodies cross-reacted with the African elephant and camel immunoglobulin, concluding the similarities of African elephant and camel immunoglobulin G (IgG) to that of other mammalian species (Azwai et al., 1993, Kelly et al., 1998).

Characterization of the IgG is a first step in developing the immunology toolbox in lions and further isolate the other antibodies and identify the immune functions of these antibodies. One end goal is to develop tools for diagnostic purposes since serology is an easy way to survey for disease. The use of serology as a tool for surveillance and epidemiological modelling of wildlife diseases has become important hence it is crucial to consider the strength and the limitations of serological assays and the interpretation of results mostly when using data for prevention and control of infectious diseases in wildlife. The ecology of infectious wildlife diseases has become critical in the animal and public health (Gilbert et al., 2013). In most cases, diseases are often diagnosed based on serological tests, as they are effective and simple (Kelly et al., 1993, El-Hewairy, 2012).

Therefore, in this study we focused on the isolation and characterization of IgG (commonly referred to as antibodies) as the major components of HI in lions using anti-cat IgG in determining the level of cross-reactivity. Sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was further used to determine the

molecular weight of lion IgG. These findings will add to the immunology toolbox in assessing the health of captive and non-captive individuals and populations.

1.5 Problem statement

The decrease in wild felid population caused by poaching, drought and diseases such as bovine tuberculosis (BTB), FIV and other microbial diseases has disrupted conservation programs (Donnelly et al., 2003, Ferreira and Funston, 2010, Maas et al., 2010). Past studies investigating these threats have shown that the Kruger National Park (KNP) has a high disease prevalence throughout various regions of the park (Antunes et al., 2008, Ferreira and Funston, 2010, Maas et al., 2010, Maas et al., 2012b, Broughton, 2017). However, there is unknown or limited information on the feline immune system and the diagnostic tests and/or tools are required to increase knowledge on wild felid immunoglobulins.

1.6 Aims and objectives

The aim of the study is to isolate and characterize lion IgG.

The objectives of the study are to:

- Determine cross-reactivity of current or available conjugates with crude and purified lion IgG.
- Purify cat and lion IgG from crude serum.
- Determine the molecular weight of lion IgG and the molecular weight of both the Fc and Fab fragments.

1.7 Hypothesis

- Goat anti-cat IgG cross-reacts with lion IgG.
- The IgG molecular weight of lion is similar to the molecular weight of cat IgG.

1.8 Scope of dissertation

The general purpose of the study is to increase information on the feline immune system. There is very little information about the IgG of lions and serological diagnostic tests. In 1998 (Kelly et al.) conducted a study on the isolation and characterization of African elephant IgG. The recent study on the hyena immunology toolbox was conducted by (Flies et al., 2012) where sera from hyena was purified. The purified IgG was then used for cross-reactivity studies and determination of the molecular

weight. In this study, the approach will be similar but limited to the characterization of the lion IgG molecule.

African lion sera from South Africa (KNP and Game Reserve in the North West Province) and Zimbabwe were used in the study. All samples were processed at the University of Pretoria, Department of Veterinary Tropical Diseases (DVTD) laboratories. The duration of the experimental work was completed in six months. In this study, a direct ELISA is used to determine the percentage of cross-reactivity against cat IgG and lion IgG using goat anti-cat IgG (H+L) antibody, horseradish peroxidase (HRP) conjugate. The level of cross-reactivity can depend on the crude serum dilutions and/or purified IgG and the dilution of the conjugate. The assay is limited to quality of samples. Fresh samples (stored for less than a year) are required for pure IgG isolation. Furthermore, the molecular weight of lion IgG is determined by using SDS-PAGE and protein molecular markers.

CHAPTER 2: MATERIAL AND METHODS

2.1 Study area

Lion serum samples were obtained from the KNP, a private nature reserve in North West province, South Africa and three private reserves in Harare, Zimbabwe as shown in figure 3. The lions in the KNP are free-ranging (roaming freely and hunting for prey) whereas they were captive (kept in confined space and fed selected meat) in Harare private reserves and in the private reserve based in North West Province.

The serum samples from a free-ranging population in the KNP were sourced from a biological bank. The serum samples from Zimbabwe were opportunistic samples and available to be used in the study with permission granted from the Department of Agriculture, Forestry and Fisheries (DAFF). The lions from a private game reserve in North West Province had a similar set up to the lions kept in Zimbabwe and serum samples were provided to contribute to the research.

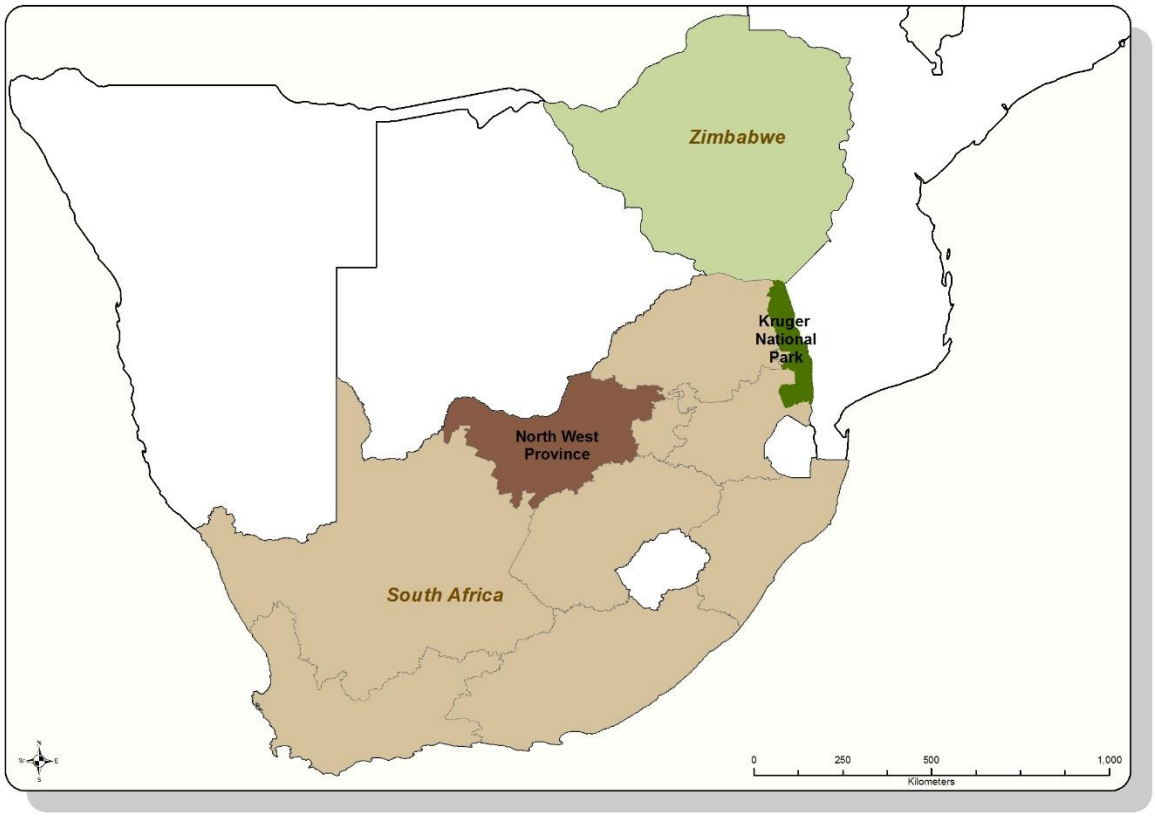

Figure 3: Map of South Africa and Zimbabwe (South African National Parks, 2020)

2.2 Ethics and biosafety

The ethics approval for the project was obtained from University of South Africa-College of Agriculture and Environmental Sciences, Animal Research Ethics Committee (UNISA-CAES) with reference number: 2018/CAES/064. Approval was also obtained from the University of Pretoria, Animal Ethics Committee (project number V023-18). The Section 20 approval for study was obtained from the Department of Agriculture, Forestry and Fisheries (DAFF) with reference number 12/11/1/1/6 (1277) and 12/11/1/1/6 (643) (see appendix).

2.3 Study design and sampling

Lion serum samples (*n*=12) from Zimbabwe were collected using 9 ml serum vacutainer tubes via venipuncture of the jugular vein. The blood samples were kept cool (on ice) during transportation to the laboratory and stored overnight in the fridge (4°C). The next day, the supernatant was separated by centrifugation at 3200 rpm for 10 minutes and stored at -80°C. Batched samples were transported to the research and training laboratories at the Department of Veterinary Tropical Diseases (DVTD), University of Pretoria, South Africa on dry ice and immediately stored at -80°C until use (Vhoko, 2018). These samples were initially collected for another study in 2016 (Vhoko, 2018) and permission was granted by the Department of Agriculture, Forestry and Fisheries (DAFF) to use these samples for this project.

The whole blood samples from all lions captured in the KNP from 2014 to 2018 (*n*=40) were kept cool (4°C) and transported to the Veterinary Wildlife Services laboratory and processed within eight hours after collection. Blood samples were centrifuged using Hermle centrifuge for 10 minutes at 2500 rpm. After centrifugation, the supernatant (serum) was dispensed in 4 ml cryo tubes using disposable pipettes then stored in freezers at -20°C and/or -80°C for future veterinary research projects. A clean disposable pipette was used for each sample to avoid cross contamination.

Whole blood from lions in a private reserve in the North West Province (*n*=16) was collected and taken to the laboratory for processing in 2019. The samples were left in the fridge for an hour before centrifugation for 10 minutes at 2800 rpm. The supernatant (serum) was then pipetted into cryo tubes and stored in a -20°C freezer for a month prior to use.

2.4 Source of domestic cat, chicken, rhinoceros and dog serum

Serum from the donor cat at the Onderstepoort Veterinary Academic Hospital was collected and used as a control in the ELISA to determine cross-reactivity. Chicken serum was obtained from the serology laboratory of the DVTD, as a control serum to include in the ELISA to determine cross-reactivity. These sources of rhinoceros and dog serum samples were included in the ELISA to determine the percentage cross-reactivity with anti-cat IgG and compare them to the % cross-reactivity of anti-cat IgG to lion IgG.

2.5 Determination of protein concentration in lion, cat and chicken crude serum

The protein concentration of lion, cat and chicken IgG in crude serum was determined using the Xpose™ Trinean Spectrophotometer. Individual samples were diluted in phosphate buffered solution (1XPBS). The concentration was determined by first diluting the serum (1:200) in PBS and then placing 2 µl of this solution on an Xpose slide. Phosphate buffered solution was used as a control. The total protein concentration (mg/ml) was determined on the Xpose™ Trinean Spectrophotometer and the absorbance of the protein was measured at 280 nm. The following calculations were used to work out the concentration of serum and IgG. For serum protein concentration, 1_{A280} = 1 mg/ml and for IgG concentration, 1.35_{A280} = 1 mg/ml. To determine the original concentration of the crude serum, the value obtained after the measurement was multiplied by the dilution factor (x200). The total protein and the serum concentration of 10 µg/ml were then used to calculate the final serum volume using the formula C_1V_1 / C_2V_2 as shown in Appendix 3.

2.6 Determining cross-reactivity of goat anti-cat IgG (H+L) secondary antibody, horseradish peroxidase (HRP) conjugate with lion IgG using lion and cat crude serum

To determine cross-reactivity and the optimal coating concentration of the crude serum, an in-house direct ELISA was developed. MaxiSorp™ (Nunc) 96-well plates with high protein binding capacity were used. These plates were coated with different concentrations of the crude serum: 10 µg/ml, 5 µg/ml, 2.5 µg/ml and 0 µg/ml. Each sample was run eight times. After coating, the plates were placed in a Biosan environmental shaker-incubator ES-20 at 150 rpm for 10 minutes at room temperature, and incubated overnight at 4°C. The coating buffer was discarded the next day, and

the plates were washed twice with a wash buffer (PBS + 0.05% Tween 20) using a wash bottle. Blocking buffer (300 µl 2% fat free milk powder in PBS + 0.05% Tween 20) was added to the wells of the plates, followed by incubation at 150 rpm for 1 hour at 37°C in a shaking incubator. After incubation, the washing step was repeated. To determine if polyclonal goat anti-cat IgG (H+L) secondary antibody, horseradish peroxidase conjugate (Invitrogen) cross-reacted with lion IgG, the conjugate used in the ELISA was diluted as follows: 1:10 000 and 1:20 000. One hundred microliter of the conjugate was added. The incubation step was repeated followed by a wash step which was performed five times using the wash buffer. The liquid substrate 3, 3′,5 ,5′-Tetramethylbenzidine (100 µl) (TMB, T4444 Sigma-Aldrich) was added to wells of the plates and incubated for two minutes. The reaction was stopped with 50 µl of 2 M Sulphuric acid (2M H_2SO_4). The plates were then read at 450 nm using Biotek Power wave XS2 plate reader and the data were exported to an Excel spreadsheet for analysis using Gen 5 software. The results were used to determine the percentage of cross-reactivity of the different species serum samples. The average mean optical density (OD) value of each test sample (lion, rhinoceros and chicken) was subtracted from the average mean OD value of the negative control (PBS) to determine the final OD. The following formula was used to determine the percentage cross-reactivity of the goat anti-cat antibody to lion IgG in crude serum. Cross-reactivity % = Optical density of test samples ÷ Optical density of the cat X 100.

Cross-reactivity % = $OD_{\text{test sample}}$ / OD_{cat} X 100

2.7 Determination of cross-reactivity of Goat Anti-cat IgG HRP conjugate with lion IgG

To determine cross-reactivity against purified lion IgG using ELISA, the following optimal conditions were used for lion and cat IgG as mentioned previously: 1µg/ml, 0.1 µg/ml, 0.01 µg/ml, 0.001 µg/ml and 0 µg/ml. A MaxiSorp™ plate was coated with purified IgG from lion and cat serum. A similar method as mentioned previously for performing ELISA was used.

2.8 Purification of cat and lion IgG from crude serum

For the purification of the IgG, a commercial kit, MagReSyn® Protein A magnetic beads (ReSyn Biosciences™ MR-PRA005, Separations) with strong binding capacity for cat IgG was used. The protocol was followed according to the manufacturer's instructions.

The MagReSyn® Protein A was supplied as a 15 mg/ml-1 suspension in TBS (50 mM), Tris pH 7.5, 150 mM NaCl with 0.025% Tween® 20 and 0.05% sodium azide as a preservative. The magnetic beads were gently vortexed using Labnet vortex mixer. The shipping solution was removed, and magnetic beads equilibrated in binding buffer (1 X PBS) before use. MagReSyn® Protein A magnetic beads were gently vortexed and 50 µl was pipetted into four 1.5 ml Eppendorf tubes. The four tubes were placed on a ReSyn Biosciences™ magnetic separator allowing the magnetic beads to clear. The supernatant was discarded. The Eppendorf tubes containing magnetic beads were placed on a rack and 300 µl of binding or wash buffer (1 X PBS) was added and incubated at room temperature for one minute, then the supernatant discarded. The washing or binding steps were repeated to a total of three washing or binding steps.

For immunoglobulin purification from lion and cat sera, nine parts binding buffer were used to dilute the pooled lion and cat serum samples. Pooled crude lion sera to increase the volume required for the purification of the lion IgG samples and cat sera were diluted each 1:10 in 1X PBS and 1 ml of this dilution was transferred to four Eppendorf tubes with the equilibrated MagReSyn Protein A, sealed with parafilm to avoid leakage and incubated at room temperature on a Labnet Rocker 25 at 90 rpm for one hour to improve binding efficiency. After incubation, the tubes were placed on a magnetic separator allowing the magnetic beads to clear, the resulting supernatants were removed and added to clean tubes before elution. These supernatants would be used to determine the binding efficiency after running the samples on SDS-PAGE. The magnetic beads were washed three times with 500 µl of 1 X PBS. Following each wash, the tubes were placed on a magnetic separator allowing the magnetic beads to clear. The supernatant from the three washes were pooled and placed in a tube labelled wash step 1 for SDS-PAGE analysis.

Elution of the captured Immunoglobulins

The captured immunoglobulins were eluted by adding 50 µl of the elution buffer (glycine pH 2.8) to the purified MagReSyn Protein A magnetic beads in four Eppendorf tubes and mixed thoroughly. The solution was incubated for 2 minutes allowing the captured IgG to elute from the magnetic beads. The tubes were placed on a magnetic separator allowing the magnetic beads to clear. The supernatants were collected in four different elution fractions (elution 1, elution 2, elution 3 and elution 4) containing

5 µl of 1 M Tris buffer pH 9.0 to neutralize the reaction. These fractions would be analysed on SDS-PAGE. The magnetic beads were washed 3 times with 1 x PBS. The supernatant from the washes were pooled and placed in a tube labelled wash step 2 for SDS-PAGE analysis.

Dialysis and concentration of samples

After the purification of lion IgG using the MegResyn A magnetic beads, 400 µl of the eluate was added to a Vivaspin Centrifugal Concentrator (VS0131 - Sartorius Vivaspin 500, 50 000 MWCO) and the eluate was centrifuged at 11 050 rcf for 10 minutes. This was then followed by the desalting step to remove the buffers used during the elution of the IgG from the magnetic beads. For the desalting step, 450 µl of PBS was added to the concentrator, then centrifuged at 11 050 rcf for 10 minutes. The desalting steps were performed three times. After the completion of the desalting steps, the final protein concentration was determined using the Xpose^{TM} Trinean Spectrophotometer. The protein was stored at 4°C until use in the SDS-PAGE analysis.

2.9 SDS-PAGE (Sodium-dodecyl sulfate polyacrylamide gel electrophoresis) analysis of the purified lion, cat, dog IgG and lion crude serum

The SDS-PAGE analysis was performed according to Laemmli (1970). The gels were prepared as shown in Table 2.

Table 2: SDS-PAGE gel preparation (Laemmli, 1970)

8% Separating Gel		Stacking Gel	
Reagents	**Volume**	**Reagents**	**Volume**
Distilled water	4.6 ml	Distilled water	3.4 ml
30% Acrylamide mix	2.7 ml	30% Acrylamide	0.83 ml
1.5M Tris (pH 8.8)	2.5 ml	0.5M Tris (pH 6.8)	0.63 ml
10% SDS	0.1 ml	10% SDS	0.05 ml
10% APS	0.1 ml	10% APS	0.05 ml
TEMED	0.006 ml	TEMED	0.005 ml
Total Volume	10 ml	Total Volume	5 ml

The gel components were mixed with ammonium persulfate (APS) and Tetramethylethylenediamine (TEMED) added before casting the gels. A gather comb was used to make a mark 1 cm beneath the comb. The acrylamide solution was

poured between the glass plates, which were clamped using a casting clamp. Drops of 100% methanol were gently added on the separating gel to break bubbles. The gel was left for 15-30 minutes to polymerize. The methanol was removed using filter paper. The stacking gel was prepared, poured on top of the polymerized separating gel, followed by placing the comb into the gel and allowing it to set for 15 to 30 minutes. The comb was gently removed before 3 µl of the two protein markers were loaded in the first two lanes, the Pink Plus Prestained Protein Ladder (Gene DireX, 500 µl volume) stored in a fridge and the Blue protein Standard Broad Range Ladder, molecular weight marker (Bio labs, 500 µl volume) stored in a freezer. Then 3 µl of each of the prepared samples were added directly into the lanes using 10 µl Eppendorf pipette and tips. The 8% SDS-PAGE gel was vertically placed in a tank with running Tris-Glycine electrophoresis buffer on ice. Electrophoresis was then carried out using Bio-Rad Mini-Protean Tetra System and Wealtec Elite 200 power supply at 60V until the dye in the sample buffer reached the bottom of the gel, approximately 1 hour and 30 minutes. The gel was gently removed from the glass plates, placed on a clear open-top flask then washed with distilled water for 15 minutes on a reciprocating shaker (FINEPCR, SH30L Reciprocating Shaker). After 15 minutes, GelCode™ Blue Stain Reagent 24590gel code blue stain (Thermo Scientific™) was used to stain the gel and placed on a reciprocating shaker for an hour. The stain was discarded safely following lab protocols for hazardous substances and the gel was gently washed with distilled water for 15 minutes on a reciprocating shaker. The gel incubated overnight at room temperature in distilled water on a reciprocating shaker for complete de-staining. The gel was then viewed on Univetec Cambridge transilluminator for visible bands, and then placed on a Biorad molecular image gel document system using the Image Lab software for analysis.

Preparation of samples

Xpose™ Trinean Spectrophotometer was used to determine the protein concentration of purified lion IgG. The final concentration of protein loaded in the wells of the SDS-PAGE was 2 µg/µl. The samples were diluted with loading buffers, one for reduced (protein solvent buffer) and the other for non-reduced (6 x agarose loading gel) samples, to a final concentration of 2 µg/µl as the optimal. The reduced, diluted samples were each placed in 2 ml Eppendorf tubes, placed on a heating block (Labnet Accublock Digital Dry Bath) at 100°C for 10 minutes and centrifuged using Wealtec E-

Centrifuge for 10 seconds at 3000 rpm. The non-reduced diluted samples were each placed in a 2 ml Eppendorf tubes. All the samples and protein markers were kept on ice during the preparation step.

CHAPTER 3: RESULTS

3.1 Determination of protein concentration in lion, cat and chicken crude serum and purified IgG

The Xpose™ Trinean Spectrophotometer software measured the protein concentration at an absorbance (OD) of 280 nm. The protein concentration (lion, cat, rhinoceros, chicken crude sera, lion IgG, cat IgG and dog IgG) was determined by the use of the Nova Biostorage Xpose™ Trinean Spectrophotometer, then further used to determine dilutions of ELISA and SDS-PAGE analysis (Appendix 3). The protein concentrations of the purified IgG were used for SDS-PAGE analysis as follows: lion IgG (39.09 mg/ml), cat IgG (2.83mg/ml), dog IgG (1.60 mg/ml) and lion crude serum (72.6mg/ml).

3.2 Determination of cross-reactivity of goat anti-cat IgG HRP conjugate with lion IgG and other species

A direct ELISA was used to determine cross-reactivity of the commercial goat anti-cat IgG (H+L) antibody to crude lion serum, as well as other animal species. All the lion and cat crude sera were included in the ELISA to determine if anti-cat antibody cross-reacted with the lion sera. The cross-reactivity was subjected to coating concentration of 10 µg/ml and 1:20 000 of the goat anti-cat IgG (H+L) antibody, HRP conjugate. As expected, the conjugate detected IgG in cat serum and cross-reacted with crude individual lion sera. High cross-reactivity was observed ranging between 87.7-100% and low reactivity with rhinoceros (22.4%) and chicken (0.01%) **(Tables 3)**.

3.3 Determination of cross-reactivity of goat Anti-cat IgG HRP conjugate with purified lion IgG

The purified IgG from pooled lion sera and purified IgG from cat serum were also tested for cross-reactivity. The goat anti-cat IgG (H+L) antibody cross-reacted with lion IgG. The percentage cross-reactivity was 93.4% for lion IgG (See **Appendix 2)**.

Table 3: Cross-reactivity of goat anti-cat immunoglobulin IgG with other animal species crude sera

Animal	OD_{450nm}	%
Domestic cat	2.2	100.0
Lion5	2.3	103.7
Lion2	2.3	101.1
Lion7	2.2	99.2
Lion3	2.2	99.2
Lion4	2.2	99.0
Lion6	2.2	98.6
Lion 1	2.2	98.4
Lion18	2.2	98.3
Lion22	2.1	95.9
Lion21	2.1	95.4
Lion23	2.1	95.1
Lion 17	2.1	93.7
Lion 20	2.1	93.2
Lion 24	2.1	92.9
Lion19	2.0	87.7
Rhinoceros	0.5	22.4
Chicken	0.0	0.01

3.4 Purification of cat and lion IgG from crude serum

Immunoglobulin G from cat and pooled lion serum samples were successfully purified and captured using MagReSyn® Protein A magnetic beads. The supernatant from before elution, the wash steps and elution fractions were read spectrophotometrically, and this indicated higher protein concentrations in the elution fraction and very low protein concentrations in both wash steps (wash step 1 and 2) as indicated in **Table 4**.

Table 4: Protein concentrations of different fractions collected during the purification step of cat and lion IgG

	CAT-APRIL	CAT-MARCH	KNP Lions	Zim Lions
	Protein (mg/ml)			
Glycine	-	-	-	-
Before Purification	9.58	-	9.67	14.6
Before Elution	3.7	-	7.72	6.88
Wash Step 1	0.1	-	0.27	0.22
Wash Step 2	0	-	0	0.01
1E1	0.02	1.82	1.06	0.69
1E2	1.88	2.23	3.11	2.98
1E3	0.5	0.58	0.73	0
2E1	-	1.88	1.09	1.55
2E2	1.25	2.39	0	2.54
2E3	0.27	0.73	0.92	0.05
3E1	2.39	3.2	2.12	1
3E2	1.69	0.73	0.58	3.01
3E3	-	0.14	0.13	0.64
4E1	0.9	2.31	1.28	1.37
4E2	0	2.24	3.14	0
4E3	0.73	0.49	0.63	0.77

3.5 Characterization of lion, cat and dog IgG on SDS-PAGE

A molecular weight marker (Blue Stain Standard Broad Range) was used in the PAGE gel to determine the molecular weight of lion IgG (Capers, 2006, Sambrook and Russell, 2006). The basic structure of Ig consists of disulphide bonds and four polypeptide chains, the two light chains (L) and two heavy chains (H) containing variable regions (V_H or V_L) at the N terminal (Neoh et al., 1973, Huse et al., 2002, Bhattacharya, 2008, Schroeder Jr and Cavacini, 2010). During SDS-PAGE analysis, heat and reducing agent (protein solvent buffer) were used to denature the proteins of

the purified IgG of lion, cat and dog. Under reducing condition, the disulphide bonds between the light and heavy chains dissociated, resulting in heavy chain band at the top and the light chain band migrating to the bottom of the lane according to the size of the polypeptide. The lion and cat IgG heavy chains were found to be in the same range (54- 56 kDa). The dog IgG heavy chain was found to be smaller than the heavy chain of both the lion and cat IgG, estimated to be 48-50 kDa. The cat IgG light chain was estimated at 26-29 kDa. It was found to be larger than the lion IgG and dog IgG light chains estimated at 24-26 kDa and 22-25 kDa. For the non-reduced samples, the disulphide bonds were reformed resulting in a single band. The band was then used to determine the molecular weight of lion IgG and compare it to the molecular weight of cat and dog IgG. The lion samples revealed a protein band estimated to be between 150-160 kDa and noted to be larger than the heavy chain for both cat and dog. The cat and dog samples produced 135-145 kDa and 100-105 kDa bands respectively under non-reducing conditions. See **Table 5** for summary of molecular weights.

Table 5: Molecular weights (kDa) of lion and other species under reduced and non-reduced conditions

	Molecular weights (kDa)		
Target	Lion	Cat	Dog
RC-Heavy chain	54-56	54-56	48-50
RC-Light chain	24-26	26-29	22-25
Non-reduced condition	150-160	135-145	100-105

***RC-Reduced conditions**

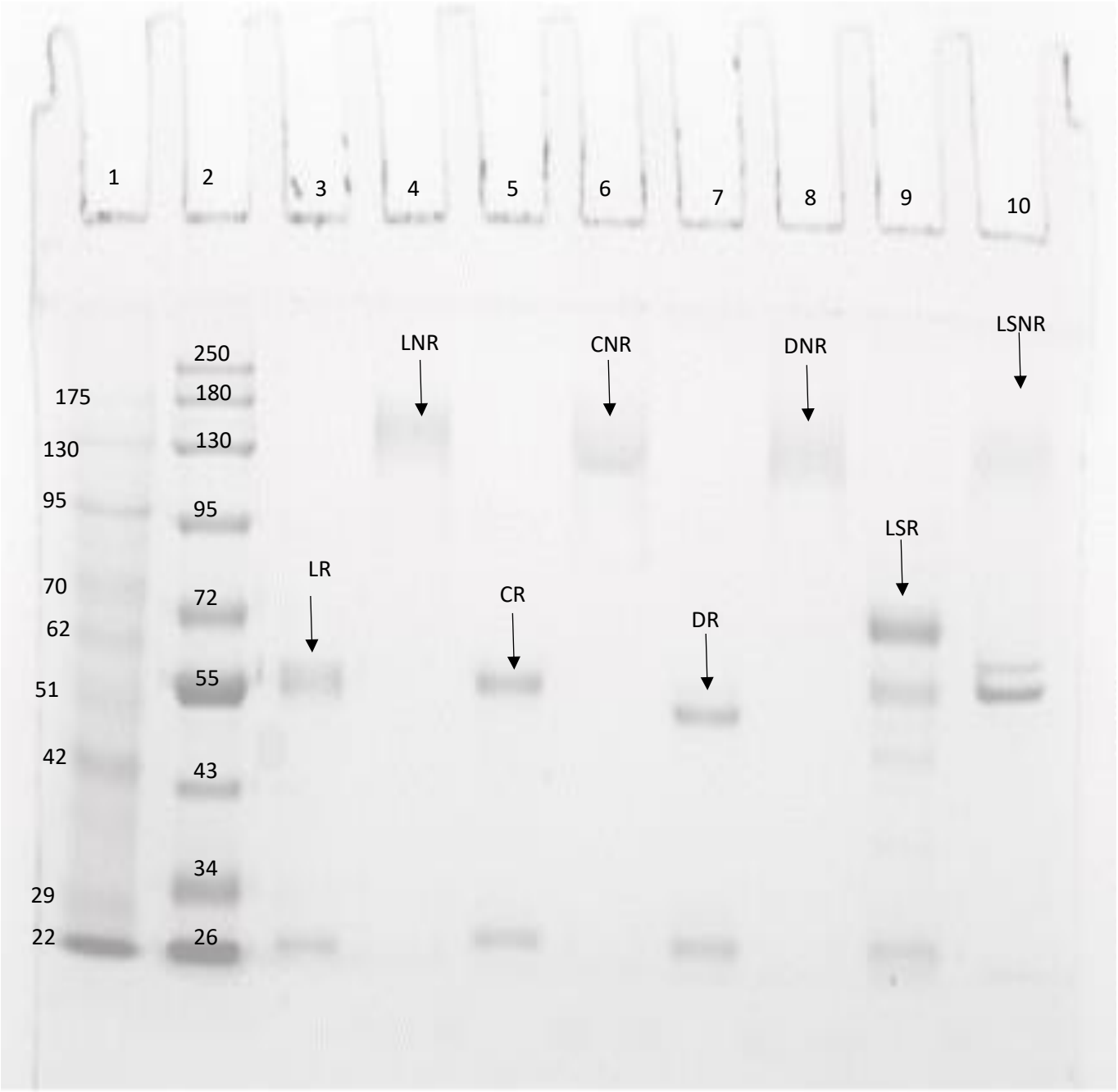

Figure 4: 8% SDS-PAGE Gel: SDS-PAGE analysis of purified lion IgG (L) compared to cat IgG (C), Dog IgG (D) and lion crude serum (LCS)

Lane 1: Pink Plus Prestained Protein Ladder, Lane 2: Blue Protein Standard Broad Range Ladder, Lane 3: Reduced Lion IgG (LR), Lane 4: Non-Reduced Lion IgG (LNR), Lane 5: Reduced Cat IgG (CR), Lane 6: Non-Reduced Cat IgG (CNR), Lane 7: Reduced Dog IgG (DR), Lane 8: Non-Reduced Dog IgG (DNR), Lane 9: Reduced Lion Sera (LSR), Lane 10: Non-Reduced Lion Sera (LSNR).

CHAPTER 4: Discussion

Ferreira and Funston (2010) have reported a decrease in wild felid populations caused by poaching, drought and infectious diseases such as BTB, FIV disease and other pathogenic diseases, whereas Green et al. (2020) have stated that there is an expansion in farms breeding lions which have created the opportunity for an increase of emerging zoonotic diseases.

In view of the above, the understanding of the immune mechanisms that may underlie disease susceptibility and provide protection against large-scale population crashes in the face of disease outbreaks has become crucial especially with the advent of the Covid-19 pandemic. According to Capers (2006), one can use the immunoglobulin purification techniques that have been used previously for purifying the immunoglobulin of other species to study the immune system of different unstudied species. Immunoglobulin IgG plays an important role in the protection against diseases and can be a good indicator of immunological responses to infectious challenges.

The aim of this study was the isolation and characterization of lion IgG as little is known about the lion immune system. Several objectives were formulated, and results obtained are discussed.

The use of the Nova Biostorage Xpose™ Trinean Spectrophotometer was used successfully to determine the protein concentration of crude serum and purified IgG. It was observed that fresh serum samples (less than a year) have higher protein concentration compared to the samples that were stored more than two years. Indeed, there are different factors that may influence the concentration levels of immunoglobulins which include age, disease condition and storage in the laboratory. The concentration of purified IgG does not deteriorate when stored at -20°C. In this study, only adult sera were used.

Cross-reactivity was observed using an in-house direct ELISA, the OD values are shown in Tables 3. A strong reactivity was observed between the goat anti-cat IgG with the lions' IgG, the highest percentage of cross-reactivity range between 87.7-100% whereas the low cross-reactivity were observed with rhinoceros (22.4%) and chicken (0.01%). These results confirm the phylogenetic relationship between domestic cat and lion. Since cat and lions are genetically related, cross-reactivity was expected at higher percentage with both crude sera and purified IgG in the study compared to the chicken sera which was included as a control sample in the ELISA. The study indicates a strong antigenic similarity between cat and lion and a more distant relationship when felids are compared with chicken. In general, species that are genetically related, show a very strong antigenic similarity and a very weak similarity if not related (Kania et al., 1997, Capers, 2006). Detecting antibodies in the sera of wild felids using anti-cat IgG is standard procedure (Penzhorn et al., 2002).

It is also worth noting that there was 22.4 % cross-reactivity with rhinoceros crude serum, although this specie is out of the felid family. These results indicate that the goat anti-cat IgG was specific to the rhinoceros to some extent. Perhaps, the antigenic determinants of IgG region of cat and rhinoceros do share homologues in the amino acid sequence of the antigen. Since rhinoceros and lion are both endangered species in South Africa, further investigation on the antigenic determinant of the immunoglobulin responsible for cross-reactivity could be explored. Immunoglobulins are useful as labelled secondary antibodies in immunoassays, it is therefore important to detect any cross-reactivity with other species to prevent false positive reactions (Ramlau, 1987).

The IgG isolated by the usage of MagReSyn® Protein A magnetic beads from ReSyn Biosciences confirmed a highly enriched IgG. The MagReSyn® Protein A has strong binding capacity for cat, dog, pig, rabbit and guinea pig IgG and was found to be effective for capturing the proteins from the lion sera and lion IgG (ReSyn-Biosciences, 2012-2017).

The results of the SDS-PAGE analysis provided us with the tool to determine the molecular mass of lion IgG. For the cat heavy chain the molecular weight is between 54-56 kDa which is in the same range (50-59 kDa) as shown in other studies (Grant, 1995, Yamada et al., 2007, Flies et al., 2012). According to the summary provided by

Flies et al. (2012), the molecular weight of cat heavy chain ranges from 50 to 59 kDa and light chains at 22 to 28 kDa. The molecular weight of dog IgG heavy and light chains ranges respectively from 50-55 kDa and 20-31 kDa (German et al., 1998, Donaghy and Moore, 2020), whereas in this study the molecular weight of both chains were respectively 48-50 kDa and 22-25 kDa. The molecular weight of lion IgG is larger than the molecular weight of cat IgG. IgG is the most common immunoglobulin with the highest concentration in serum and plays a major role in the antibody-mediated defence mechanisms as a type of passive immunity (Tizard Ian, 1996, Bhattacharya, 2008). The lion immunoglobulin is also found to be closely similar to the immunoglobulins of other Felidae (domestic cat) and Canidae (spotted hyena and domestic dog) (Yamada et al., 2007, Flies et al., 2012).

SDS-PAGE is the simplest and least expensive and most used technique to analyse antibodies for purity. On SDS-PAGE, the reduced samples of IgG class of antibodies provide heavy chains of approximately 50 kDa and light chains of approximately 25 kDa. When these samples are analysed without reduction of disulphide bonds, the antibodies should give rise to a single band where the intact antibody consisting of two heavy and two light chains, with a combined size of approximately 150 kDa (Kirley and Norman, 2018). In this study, the non-reduced lion IgG sample size of the molecule is estimated to be between 150-160 kDa.

Tizard Ian (1996) stated that IgG is the smallest of the immunoglobulin classes with a molecular weight of 180 kDa and can easily escape from blood vessels and play an important role in passive immunity. Our results have shown that the molecular weight of the IgG is 150-160 kDa. This may suggest that the lion maternal passive immunity to the foetus within natural habitat might be high.

SDS-PAGE has been widely used to characterize IgG of other mammalian species and the results have been proven to be consistent with the results of western blotting technique (Capers, 2006, Yamada et al., 2007, Flies et al., 2012). To our knowledge, besides the study conducted by Flies et al. (2012) on hyena immunology, there are no studies that determined the molecular weight of lion IgG.

Basic immunology tools can be used in studying the immune function in different species to determine the exposure to an infectious disease even during disease outbreaks.

CHAPTER 5: Conclusions, Research challenges and Future perspectives

5.1 Conclusions

The objectives of the study as set out were achieved within the time constraint.

5.1.1 Determine cross-reactivity of current or available antibodies with lion IgG.

The concentration of protein in the crude sera from different species was determined. There was cross-reactivity, high similarity was observed between cat and lion. The percentage of cross-reactivity dropped with other species as the species are not genetically related to lion.

5.1.2 Purify cat and lion IgG from crude serum.

The usage of the MagReSyn® Protein A magnetic beads from ReSyn Biosciences was effective for purification of lion IgG with a superior binding capacity. Cross-reactivity of purified IgG and crude sera was observed on ELISA using Goat anti-cat IgG.

5.1.3 Determine the molecular weight of lion IgG and the molecular weight of both the Fc and Fab fragments.

The molecular weight from purified lion Ig was characterized on SDS-PAGE and successfully measured. The lion and cat IgG heavy chain were found to be in the same range (54- 56 kDa). The dog IgG was slightly smaller than the heavy chain of both the lion IgG and cat IgG, estimated to be 48-50 kDa.

5.2 Research challenges

- Old sera did not yield any protein most likely due to denaturation of proteins during a long storage whereas the fresh sera yielded a high concentration of proteins (IgG) which allowed the characterization of proteins.
- The isolation and characterization of the lion immunoglobulin was laborious and difficult process, the handling of the gel was a delicate technique to master.
- Lion IgG is large and could not be fully characterized using SDS PAGE.

5.3 Future perspectives

5.3.1. There are some activities that have derived from this research, which will be carried out for publication purposes. These activities will be planned as short, medium and long-term goals.

5.3.2. All bioassays related to this research project were conducted *in vitro*. There is still a need for the development of anti-lion antibodies *in vivo* using laboratory animals/other small animal species as these anti-lion antibodies are not commercially available.

5.3.3. Isolation and sequencing of whole IgG genome from lions in captivity and semi-captivity.

5.3.4. Comparing immunoglobulins profile of captive and/or free-ranging lions from neighbouring countries of South Africa.

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7. APPENDICES

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Isolation and characterization of immunoglobulins from lion (*Panthera leo)* from Zimbabwe and South Africa**
PROJECT NUMBER	**V023-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. D Morar-Leather & T Manamela**

STUDENT NUMBER (where applicable)	**U_29228132**
DISSERTATION/THESIS SUBMITTED FOR	**Academic & MSc (UNISA)**

ANIMAL SPECIES	**Lion**
NUMBER OF SAMPLES	**40 (See V055-16)**
Approval period to use animals for research/testing purposes	**April 2018 - April 2019**
SUPERVISOR	**Dr. P Kayoka-Kabongo**

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 23 April 2018
CHAIRMAN: UP Animal Ethics Committee	Signature Nico Bennett

S4285-15

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

08 May 2018

TO THE ANIMAL ETHICS COMMITTEE

RE: CAT SERUM SAMPLES

Dr Darshana Morar-Leather has requested cat serum samples from the Onderstepoort Veterinary Academic Hospital (OVAH) to be used in an MSc research project titled "Isolation and characterization of immunoglobulin from *Panthera leo* from Zimbabwe and South Africa". The cat serum samples will be used as controls in the study.

I give my consent and she may contact the veterinarians in the small animal clinic to obtain the samples required for the study.

Yours sincerely

PROF M HARTMAN
ACTING DIRECTOR - OVAH

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/6 (643)

Ms Tebogo Sabina Manamela
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria
Tel: 012 529 8278
E-mail: tbgmanamela@gmail.com; darshana.morar@up.ac.za

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Dear Ms Manamela,

Your application sent with the email on 29 November 2017 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;
3. The specified lion serum samples from Zimbabwe (section 20 approval reference no. 12/11/1/1/8) stored at the DVTD may be utilised for the study as indicated;
4. The specified lion serum samples from the Veterinary Wildlife Services (VWS) Laboratory may be utilised for the study as indicated. Based on the letter received from the VWS senior manager and the background information on the specified samples, the samples are regarded as low risk samples and may be transported to

the Department of Veterinary Tropical Diseases. Samples must be packaged and transported in accordance with the National Road Traffic Act, 1996 (Act No. 93 of 1996) and must travel under the cover of Red Cross Permit issued by the State Veterinarian of the area of origin and in full compliance with all the conditions of such a permit;

5. All samples and potentially infectious material utilised, collected or generated during the study is to be destroyed at the completion of the study. Records must be kept for five years for auditing purposes.
6. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval;

Title of research/study: "*Isolation and characterisation of immunoglobulins from Panthera leo in southern Africa*".
Researcher: Ms Tebogo Sabina Manamela
Institution: Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria
Our ref Number: 12/11/1/1/6 (643)
Your ref: -
Expiry date: December 2018

Kind regards,

ACTING DIRECTOR OF ANIMAL HEALTH
Date:
2018 -01- 12

SUBJECT: *PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)*

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/6 (1277)

Ms Tebogo Sabina Manamela
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria
Tel: 012 529 8278
E-mail: tbgmanamela@gmail.com; darshana.morar@up.ac.za

RE: AMENDMENT OF SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "ISOLATION AND CHARACTERISATION OF IMMUNOGLOBULINS FROM PANTHERA LEO IN SOUTHERN AFRICA".

An amendment is hereby granted on the Section 20 approval that was issued for the above mentioned study on 12 January 2018 (attached):

i. 17 additional lion serum samples may be sourced from Ukutula Private Game farm in the North West Province, as indicated. It is the researcher's responsibility to remain in contact with the responsible State Veterinarian regarding the disease status of the area during sampling. Records must be kept for five years for auditing purposes;

ii. As requested, the validity of the section 20 approval is extended to December 2019;

iii. Remaining lion serum from the additional Ukutula samples may be stored at -80°C under access control in the DVTD Research and Training Laboratory for the duration of the study until a permanent biobank has been inspected and approved. Stored samples may not be used for further research or be outsourced without prior written approval from the Director: Animal Health;

iv. All conditions as specified in the Section 20 approval of 12 January 2018 remain in effect.

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2019 -10- 22

UNISA university of south africa

UNISA-CAES ANIMAL RESEARCH ETHICS COMMITTEE

Date: 10/10/2019

NHREC Registration # : N/A
ERC Reference # : 2018/CAES/064
Name : Ms TS Manamela
Student #: 61025682

Dear Ms Manamela

Decision: Ethics Approval Renewal after First Review from 01/06/2019 to 31/05/2020

Researcher(s): Ms TS Manamela
tbgmanamela@gmail.com

Supervisor (s): Dr P Kayoka-Kabongo
kabonpnk@unisa.ac.za; 011-471-2949

Dr D Morar-Leather
Darshana.morar@up.ac.za; 012-529-8312

Working title of research:

Isolation and characterization of immunoglobulins from *Panthera leo* in Southern Africa

Qualification: MSc Agriculture

Thank you for the submission of your progress report to the Unisa-CAES Animal Research Ethics Committee for the above mentioned research. Ethics approval is renewed for a one-year period. After one year the researcher is required to submit a progress report, upon which the ethics clearance may be renewed for another year.

Due date for progress report: 31 May 2020

Please note the following for further action:

1. *The researcher is cautioned to adhere to the stipulations of the Section 20 permit. Should any changes to the design of the research project be necessary, an updated Section 20 permit needs to be obtained and submitted to the Committee.*

University of South Africa
Preller Street, Muckleneuk Ridge, City of Tshwane
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*The **medium risk application** was **reviewed** by the UNISA-CAES Animal Research Ethics Committee on 29 May 2018 in compliance with the Unisa Policy on Research Ethics and the Standard Operating Procedure on Research Ethics Risk Assessment.*

The proposed research may now commence with the provisions that:

1. The researcher(s) will ensure that the research project adheres to the values and principles expressed in the UNISA Policy on Research Ethics.
2. Any adverse circumstance arising in the undertaking of the research project that is relevant to the ethicality of the study should be communicated in writing to the Committee.
3. The researcher(s) will conduct the study according to the methods and procedures set out in the approved application.
4. Any changes that can affect the study-related risks for the research participants, particularly in terms of assurances made with regards to the protection of participants' privacy and the confidentiality of the data, should be reported to the Committee in writing, accompanied by a progress report.
5. The researcher will ensure that the research project adheres to any applicable national legislation, professional codes of conduct, institutional guidelines and scientific standards relevant to the specific field of study. Adherence to the following South African legislation is important, if applicable: Protection of Personal Information Act, no 4 of 2013; Children's act no 38 of 2005 and the National Health Act, no 61 of 2003.
6. Only de-identified research data may be used for secondary research purposes in future on condition that the research objectives are similar to those of the original research. Secondary use of identifiable human research data require additional ethics clearance.
7. No field work activities may continue after the expiry date. Submission of a completed research ethics progress report will constitute an application for renewal of Ethics Research Committee approval.

Note:
*The reference number **2018/CAES/064** should be clearly indicated on all forms of communication with the intended research participants, as well as with the Committee.*

Yours sincerely,

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Dr WM Strauss
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Appendix 1: Lion (KNP and Zimbabwe) and Cat Elution Fractions from crude serum

	Position	Sample name	Protein (mg/ml)	E1%	Impurities (A280)	Background (A280)	Residue (%)	A280	A260/A280
Glycine	A1	blank_A1	-	-	-	0.08	-	0	-
E1 Cat	B1	sample_B1	-	10	-	-	-	-	-
E2 Cat	C1	sample_C1	1.56	10	0.01	0.08	0.4	1.59	0.53
E3 Cat	D1	sample_D1	1.8	10	0	0.21	0.6	1.8	0.52
E4 Cat	E1	sample_E1	1.65	10	0	0.37	0.5	1.66	0.53
E1 Lion	F1	sample_F1	1.55	10	0.01	0.28	0.6	1.58	0.51
E2 Lion	G1	sample_G1	1.91	10	0.22	0.01	1.6	2.15	0.58
E3 Lion	H1	sample_H1	1.58	10	0.01	0.11	0.8	1.6	0.52
E4 Lion	A2	sample_A2	1.64	10	0.01	0.4	0.4	1.66	0.53

Appendix 2: ELISA results for Cross-reactivity between cat and lion IgG

	Cat								Lion							
Dilutions	**1**	**2**	**3**	**4**	**5**	**6**	**μ**	**OD**	**7**	**8**	**9**	**10**	**11**	**12**	**μ**	**OD**
1 μg/ml	2.619	2.568	2.487	2.54	2.531	2.557	2.550	2.463	2.366	2.324	2.379	2.332	2.329	2.387	2.353	2.300
0.1 μg/ml	1.491	1.403	1.473	1.473	1.456	1.424	1.453	1.366	1.152	1.103	1.093	1.127	1.159	1.188	1.137	1.085
0.01 μg/ml	0.299	0.287	0.3	0.282	0.31	0.303	0.297	0.209	0.198	0.205	0.212	0.204	0.215	0.226	0.210	0.158
0.001 μg/ml	0.128	0.09	0.092	0.078	0.145	0.083	0.103	0.015	0.067	0.061	0.063	0.061	0.062	0.061	0.063	0.010
0 μg/ml	0.146	0.07	0.081	0.058	0.112	0.059	0.088	0.000	0.079	0.049	0.049	0.049	0.045	0.044	0.053	0.000

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Appendix 3: Summary of protein concentration and cross-reactivity of lions, cat and chicken crude serum.

Sample Name	Protein (mg/ml)	Total Protein (mg/ml)	Final Volume	Final Volume (ml)	Anti-cat ELISA Average	Repeats Anti-cat ELISA Average	OD
Lion 1	0.39	78	0.26	2	2.25	2.07	2.2
Lion 2	0.42	84	0.24	2	2.31	0	2.26
Lion 3	0.55	110	0.45	5	2.27	0	2.22
Lion 4	0.41	82	0.24	2	2.26	0	2.21
Lion 5	0.46	92	0.22	2	2.37	0	2.32
Lion 6	0.42	84	0.24	2	2.25	0	2.21
Lion 7	0.36	72	0.28	2	2.27	0	2.22
Lion 8	0.25	50	0.4	2	2.24	0	2.19
Lion 9	0.34	68	0.29	2	2.28	0	2.23
Lion 10	0.38	76	0.26	2	2.25	0	2.2
Lion 11	0.33	66	0.3	2	2.23	0	2.19
Lion 12	0.35	70	0.29	2	2.24	0	2.19
Lion 13	0.49	98	0.2	2	2.3	0	2.25
Lion 14	0.42	84	0.24	2	2.18	0	2.14
Lion 15	0.39	78	0.26	2	2.24	0	2.19
Lion 16	0.34	68	0.29	2	2.28	0	2.24
Lion 17	0.33	66	0.3	2	2.14	0	2.1
Lion 18	0.51	102	0.49	5	2.24	0	2.2
Lion 19	0.31	62	0.32	2	2.01	0	1.96
Lion 20	0.36	72	0.28	2	2.13	0	2.09
Lion 21	0.25	50	0.4	2	2.18	0	2.13

Lion 22	0.25	50	0.4	2	2.19	0	2.15
Lion 23	0.33	66	0.3	2	2.17	0	2.13
Lion 24	0.33	66	0.3	2	2.12	0	2.08
Lion 25	0.53	106	0.47	5	2.28	0	2.23
Lion 26	0.38	76	0.26	2	2.28	0	2.23
Lion 27	0.38	76	0.26	2	2.28	0	2.24
Lion 28	0.4	80	0.25	2	2.33	0	2.28
Lion 29	0.32	64	0.31	2	2.41	0	2.36
Lion 30	0.41	82	0.24	2	2.4	0	2.36
Lion 31	0.34	68	0.29	2	2.27	0	2.33
Lion 32	0.22	44	0.45	2	1.39	1.32	1.34
Lion 33	0.33	66	0.3	2	2.3	0	2.25
Lion 34	0.48	96	0.21	2	2.33	0	2.28
Lion 35	0.48	96	0.21	2	2.27	0	2.22
Lion 36	0.36	72	0.28	2	2.27	0	2.22
Lion 37	0.43	86	0.23	2	2.26	0	2.21
Lion 38	0.5	100	0.2	2	2.33	0	2.28
Lion 39	0.44	88	0.23	2	2.28	0	2.23
Lion 40	0.36	72	0.28	2	2.26	0	2.21
ZimLion 1	1.55	310	0.32	10	1.74	2.02	1.96
ZimLion 2	0.41	82	0.24	2	1.86	2.1	2.04
ZimLion 3	0.4	80	0.25	2	1.91	2.09	2.03
ZimLion 4	0.54	108	0.46	5	1.85	2.03	1.97
ZimLion 5	0.59	118	0.42	5	1.89	2.03	1.98
ZimLion 6	0.45	90	0.2	2	1.99	2.13	2.08
ZimLion 7	0.62	124	0.4	5	1.77	1.98	1.92
ZimLion 10	0.37	74	0.27	2	1.67	1.86	1.8
ZimLion 11	0.33	66	0.3	2	1.87	1.93	1.87
ZimLion 12	0.43	86	0.23	2	1.86	1.93	1.88

ZimLion 13	0.63	126	0.4	5	1.83	1.91	1.85
ZimLion 14	0.36	72	0.28	2	1.87	1.93	1.87
Cat	0.25	50	0.8	2	2.14	0	2.14
Rhino	0.9	180	0.27	5	0.51	0	0.5
Chicken	0.25	50	0.8	2	0.05	0	0
PBS	0	0	0	0	0.05	0	0

Appendix 4: ELISA results for Cross-reactivity between cat, lion, chicken and rhino crude serum

10 µg/ml												
1:20 000 Conjugate	**Cat**	**Rhino**	**Chicken**	**Lion1**	**Lion 2**	**Lion 3**	**Lion 4**	**Lion5**	**Lion6**	**Lion7**	**Lion8**	**Negative**
A	2.22	0.55	0.05	2.28	2.25	2.32	2.27	2.30	2.29	2.28	2.30	0.05
B	2.27	0.54	0.05	2.17	2.35	2.31	2.23	2.34	2.24	2.30	2.27	0.04
C	2.28	0.55	0.05	2.28	2.29	2.23	2.14	2.53	2.21	2.21	2.23	0.05
D	2.19	0.53	0.05	2.15	2.33	2.18	2.15	2.22	2.28	2.29	2.16	0.04
E	2.30	0.54	0.05	2.22	2.28	2.19	2.34	2.23	2.21	2.21	2.20	0.05
F	2.40	0.56	0.05	2.26	2.27	2.28	2.40	2.71	2.19	2.28	2.21	0.05
G	2.36	0.55	0.05	2.29	2.33	2.28	2.27	2.28	2.29	2.32	2.21	0.07
H	2.35	0.57	0.05	2.34	2.38	2.37	2.30	2.34	2.34	2.26	2.35	0.04
Mean	**2.2951**	**0.5475**	**0.0471**	**2.2494**	**2.3118**	**2.2683**	**2.2628**	**2.3685**	**2.2548**	**2.2686**	**2.2419**	**0.0490**
OD	**2.2461**	**0.4985**	**-0.0019**	**2.2004**	**2.2628**	**2.2193**	**2.2138**	**2.3195**	**2.2058**	**2.2196**	**2.1929**	**0.0000**

10 µg/ml												
1:20 000 Conjugate	**Cat**	**Rhino**	**Chicken**	**Lion 9**	**Lion 10**	**Lion 11**	**Lion 12**	**Lion 13**	**Lion 14**	**Lion 15**	**Lion 16**	**Negative**
A	2.25	0.55	0.05	2.28	2.43	2.34	2.35	2.35	2.21	2.32	2.19	0.04
B	2.30	0.55	0.05	2.22	2.22	2.48	2.22	2.25	2.24	2.23	2.13	0.04
C	2.28	0.54	0.05	2.16	2.24	2.22	2.21	2.21	2.08	2.21	2.21	0.05
D	2.21	0.52	0.05	2.30	2.14	2.17	2.14	2.28	2.16	2.20	2.22	0.05

E	2.28	0.54	0.05	2.31	2.13	2.19	2.20	2.22	2.14	2.19	2.50	0.06
F	2.30	0.55	0.05	2.29	2.38	2.19	2.23	2.35	2.21	2.18	2.47	0.05
G	2.24	0.54	0.05	2.30	2.16	2.11	2.23	2.33	2.18	2.25	2.27	0.04
H	2.31	0.55	0.04	2.37	2.29	2.18	2.32	2.39	2.22	2.32	2.29	0.03
Mean	**2.2716**	**0.5414**	**0.0476**	**2.2794**	**2.2464**	**2.2344**	**2.2371**	**2.2978**	**2.1803**	**2.2364**	**2.2824**	**0.0449**
OD	**2.2268**	**0.4965**	**0.0028**	**2.2345**	**2.2015**	**2.1895**	**2.1923**	**2.2529**	**2.1354**	**2.1915**	**2.2375**	**0.0000**

10 µg/ml												
1:20 000 Conjugate	**Cat**	**Rhino**	**Chicken**	**Lion 17**	**Lion 18**	**Lion 19**	**Lion 20**	**Lion 21**	**Lion 22**	**Lion 23**	**Lion 24**	**Negative**
A	2.33	0.57	0.05	2.19	2.32	2.09	2.17	2.22	2.29	2.25	2.10	0.05
B	2.27	0.55	0.05	2.23	2.31	2.06	2.15	2.98	2.23	2.19	2.10	0.05
C	2.31	0.53	0.05	2.19	2.32	2.04	2.16	2.11	2.24	2.22	2.14	0.04
D	2.23	0.56	0.05	2.11	2.21	2.01	2.17	2.05	2.07	2.12	2.13	0.04
E	2.22	0.54	0.05	2.07	2.26	1.96	2.09	1.98	2.10	2.21	2.12	0.04
F	2.26	0.55	0.05	2.16	2.14	1.93	2.13	2.02	2.23	2.05	2.14	0.04
G	2.27	0.52	0.05	2.07	2.16	1.97	2.05	2.03	2.17	2.15	2.13	0.05
H	2.30	0.56	0.05	2.11	2.22	1.99	2.12	2.06	2.21	2.20	2.14	0.04
Mean	**2.2728**	**0.5469**	**0.0469**	**2.1410**	**2.2434**	**2.0068**	**2.1300**	**2.1783**	**2.1909**	**2.1728**	**2.1226**	**0.0446**
OD	**2.2281**	**0.5023**	**0.0023**	**2.0964**	**2.1988**	**1.9621**	**2.0854**	**2.1336**	**2.1463**	**2.1281**	**2.0780**	**0.0000**

10 µg/ml												
1:20 000 Conjugate	**Cat**	**Rhino**	**Chicken**	**Lion 25**	**Lion 26**	**Lion 27**	**Lion 28**	**Lion 29**	**Lion 30**	**Lion 31**	**Lion 32**	**Negative**
A	2.43	0.61	0.05	2.39	2.31	2.33	2.38	2.48	2.52	2.39	0.67	0.04
B	2.40	0.59	0.05	2.23	2.34	2.33	2.34	2.32	2.33	2.35	1.52	0.04
C	2.47	0.60	0.05	2.33	2.27	2.42	2.37	2.38	2.62	2.30	1.47	0.05
D	2.48	0.58	0.05	2.27	2.23	2.26	2.41	2.49	2.32	2.37	1.53	0.05
E	2.30	0.58	0.05	2.28	2.22	2.23	2.31	2.41	2.32	2.25	1.44	0.05
F	2.38	0.59	0.05	2.23	2.31	2.24	2.32	2.41	2.35	2.50	1.41	0.06

G	2.41	0.57	0.05	2.23	2.29	2.25	2.23	2.47	2.38	2.37	1.59	0.05
H	2.45	0.58	0.06	2.28	2.24	2.21	2.25	2.30	2.37	2.47	1.46	0.05
Mean	**2.4140**	**0.5860**	**0.0499**	**2.2778**	**2.2754**	**2.2836**	**2.3256**	**2.4064**	**2.4016**	**2.3731**	**1.3856**	**0.0489**
OD	**2.3694**	**0.5414**	**0.0053**	**2.2331**	**2.2308**	**2.2390**	**2.2810**	**2.3618**	**2.3570**	**2.3285**	**1.3410**	**0.0043**

10 µg/ml

1:20 000 Conjugate	**Cat**	**Rhino**	**Chicken**	**Lion 33**	**Lion 34**	**Lion 35**	**Lion 36**	**Lion 37**	**Lion 38**	**Lion 39**	**Lion 40**	**Negative**
A	2.25	0.57	0.06	2.40	2.36	2.31	2.34	2.35	2.35	2.32	2.25	0.04
B	2.24	0.51	0.04	2.24	2.28	2.21	2.29	2.25	2.33	2.26	2.29	0.05
C	2.20	0.51	0.04	2.28	2.39	2.18	2.27	2.25	2.31	2.30	2.31	0.07
D	2.20	0.52	0.04	2.20	2.42	2.21	2.29	2.27	2.34	2.28	2.26	0.04
E	2.17	0.53	0.04	2.40	2.23	2.20	2.21	2.18	2.28	2.26	2.20	0.05
F	2.17	0.51	0.04	2.36	2.38	2.23	2.30	2.36	2.38	2.28	2.23	0.05
G	2.17	0.57	0.04	2.29	2.26	2.58	2.22	2.15	2.28	2.25	2.29	0.06
H	2.16	0.51	0.04	2.25	2.31	2.24	2.25	2.29	2.34	2.30	2.28	0.04
Mean	**2.1951**	**0.5298**	**0.0414**	**2.3016**	**2.3290**	**2.2690**	**2.2706**	**2.2626**	**2.3259**	**2.2795**	**2.2641**	**0.0499**
OD	**2.1453**	**0.4799**	**-0.0085**	**2.2518**	**2.2791**	**2.2191**	**2.2208**	**2.2128**	**2.2760**	**2.2296**	**2.2143**	**0.0000**

10 µg/ml

1:20 000 Conjugate	**Cat**	**Rhino**	**Chicken**	**ZimLion 1**	**ZimLion 2**	**ZimLion 3**	**ZimLion 4**	**ZimLion 5**	**ZimLion 6**	**ZimLion 7**	**ZimLion 10**	**Negative**
A	1.81	0.43	0.04	1.80	1.83	1.88	1.79	1.85	1.93	1.77	1.65	0.05
B	1.89	0.41	0.04	1.72	1.90	1.88	1.97	1.92	1.92	1.77	1.63	0.04
C	1.88	0.42	0.04	1.75	1.94	1.88	1.80	1.87	1.90	1.77	1.63	0.05
D	1.80	0.42	0.04	1.72	1.85	1.96	1.87	1.86	1.99	1.74	1.72	0.04
E	1.80	0.42	0.04	1.77	1.81	1.95	1.81	1.87	2.01	1.78	1.66	0.05
F	1.83	0.44	0.04	1.74	1.83	1.90	1.80	1.87	1.98	1.79	1.74	0.05
G	1.81	0.42	0.04	1.71	1.83	1.90	1.87	1.95	2.17	1.75	1.66	0.04
H	1.81	0.42	0.04	1.74	1.85	1.95	1.87	1.96	2.00	1.79	1.73	0.04
Mean	**1.8288**	**0.4226**	**0.0393**	**1.7421**	**1.8550**	**1.9113**	**1.8469**	**1.8939**	**1.9866**	**1.7701**	**1.6740**	**0.0460**

OD	**1.7828**	**0.3766**	**-0.0067**	**1.6961**	**1.8090**	**1.8653**	**1.8009**	**1.8479**	**1.9406**	**1.7241**	**1.6280**	**0.0000**

10 µg/ml												
1:20 000 Conjugate	**Cat**	**Rhino**	**Chicken**	**ZimLion 11**	**ZimLion 12**	**ZimLion 12**	**ZimLion 13**	**ZimLion 13**	**ZimLion 14**	**ZimLion 14**	**Lion 32**	**Negative**
A	2.08	0.47	0.04	2.00	1.88	1.83	1.85	1.81	1.89	1.82	1.22	0.04
B	1.95	0.45	0.15	1.89	1.76	1.82	1.82	1.80	1.87	1.74	1.16	0.03
C	2.04	0.46	0.04	1.84	1.87	1.80	1.77	1.80	1.79	2.02	1.17	0.04
D	2.04	0.48	0.03	1.79	1.81	1.83	1.79	1.75	1.81	1.82	1.21	0.04
E	2.01	0.49	0.04	1.82	1.82	1.83	1.81	2.20	1.81	1.86	1.20	0.05
F	1.98	0.47	0.04	1.82	1.91	1.95	1.74	1.80	1.92	1.84	1.26	0.07
G	1.98	0.47	0.04	1.90	1.87	1.87	1.79	1.79	1.82	2.03	1.24	0.05
H	2.02	0.52	0.04	1.93	1.91	1.96	1.86	1.84	2.00	1.91	1.28	0.05
Mean	**2.0124**	**0.4749**	**0.0514**	**1.8740**	**1.8520**	**1.8610**	**1.8023**	**1.8473**	**1.8624**	**1.8790**	**1.217**	**0.045**
OD	**1.9674**	**0.4299**	**0.0064**	**1.8290**	**1.8070**	**1.8160**	**1.7573**	**1.8023**	**1.8174**	**1.8340**	**1.1720**	**0.0000**

Plate 13 anti-cat ELISA		1	2	3	4	5	6	7	8	9	10	11	12
1:20 000 conjugate		cat serum	rhino	Chicken	Lion 1	Lion 32	ZimLion 1	ZimLion 2	ZimLion 3	ZimLion 4	ZimLion 5	ZimLion 6	Negative
A	10 µg/ml	1.99	0.50	0.06	2.22	1.37	2.06	2.15	2.17	2.08	2.07	2.14	0.05
B	10 µg/ml	2.04	0.50	0.05	2.16	1.33	2.07	2.17	2.17	2.05	2.05	2.13	0.05
C	10 µg/ml	2.00	0.49	0.05	0.05	1.33	2.04	2.12	2.12	2.07	2.08	2.12	0.04
D	10 µg/ml	2.03	0.46	0.06	2.14	1.32	2.01	2.13	2.08	2.03	2.01	2.11	0.10
E	10 µg/ml	1.99	0.48	0.06	2.07	1.30	2.02	2.14	2.04	1.99	1.98	2.11	0.05
F	10 µg/ml	2.04	0.47	0.05	1.97	1.32	2.07	2.02	2.03	2.03	2.03	2.25	0.05
G	10 µg/ml	1.98	0.46	0.05	2.06	1.28	1.93	2.01	2.00	1.99	2.03	2.11	0.05
H	10 µg/ml	2.03	0.48	0.06	2.07	1.29	1.93	2.04	2.09	1.98	2.03	2.12	0.05
Mean	10 µg/ml	**2.0133**	**0.4796**	**0.0526**	**2.0985**	**1.3161**	**2.0154**	**2.0978**	**2.0878**	**2.0266**	**2.0341**	**2.1349**	**0.0538**
OD	10 µg/ml	**1.9595**	**0.4259**	**-0.0011**	**2.0448**	**1.2624**	**1.9616**	**2.0440**	**2.0340**	**1.9729**	**1.9804**	**2.0811**	**0.0000**

Plate 14 anti-cat ELISA		1	2	3	4	5	6	7	8	9	10	11	12
1:20 000 conjugate		cat serum	rhino	Chicken	Lion 1	Lion32	ZimLion 7	ZimLion 10	ZimLion 11	ZimLion 12	ZimLion 13	ZimLion 14	Negative
A	10 µg/ml	2.02	0.48	0.04	2.02	1.29	1.97	1.87	1.94	1.91	1.87	1.91	0.06
B	10 µg/ml	2.00	0.47	0.05	2.01	1.29	2.33	1.84	1.96	1.93	1.83	1.90	0.05
C	10 µg/ml	1.98	0.48	0.07	2.00	1.32	1.94	1.87	1.94	1.91	1.89	1.89	0.05
D	10 µg/ml	1.99	0.48	0.05	2.04	1.36	1.91	1.86	1.94	1.92	1.86	1.88	0.05
E	10 µg/ml	2.01	0.49	0.05	2.05	1.33	1.90	1.87	1.94	2.06	1.88	1.97	0.06
F	10 µg/ml	2.05	0.49	0.05	2.04	1.34	1.94	1.88	1.89	1.94	2.13	2.00	0.05
G	10 µg/ml	1.99	0.47	0.05	2.02	1.31	1.93	1.82	1.90	1.88	1.88	1.95	0.06
H	10 µg/ml	1.99	0.47	0.04	2.04	1.32	1.92	1.86	1.93	1.93	1.89	1.93	0.08
Mean	10 µg/ml	**2.0028**	**0.4769**	**0.0489**	**2.0270**	**1.3199**	**1.9799**	**1.8593**	**1.9299**	**1.9348**	**1.9056**	**1.9294**	**0.0575**
OD	10 µg/ml	**1.9453**	**0.4194**	**-0.0086**	**1.9695**	**1.2624**	**1.9224**	**1.8018**	**1.8724**	**1.8773**	**1.8481**	**1.8719**	**0.0000**