Screening of captive lions (*Panthera leo*) for selected infectious diseases of significance in Zimbabwe

by

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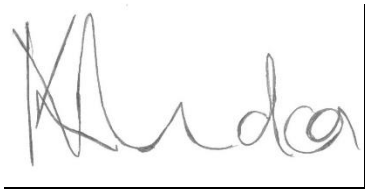
Declaration

I, Kudzaishe Vhoko, student number U15390692, hereby declare that this dissertation, "*Screening of captive lions (Panthera leo) for selected infectious diseases of significance in Zimbabwe,*" is submitted in accordance with the requirements for the Master of Science degree at University of Pretoria, is my own original work and has not previously been submitted to any other institution of higher learning. This is not a plagiarised document. All sources cited or quoted in this research paper are indicated and acknowledged with a comprehensive list of references.



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Abstract

Fifteen lions from three captive/semi-captive establishments around Harare were captured for blood sampling. Routine health checks for viral diseases and other diseases of economic, public health or biodiversity conservation importance had never been done at the time the study was carried out. The serum samples were screened against feline immunodeficiency virus, feline leukaemia virus and canine distemper virus. All samples tested negative. This screening exercise was important in order to reduce the confounding effect of these infections on tests that utilise cell-mediated immune responses, such as the interferon-gamma (IFN-γ) assay for bovine tuberculosis (BTB) diagnosis.

Whole blood processing using two antigens and three mitogens was carried out on samples collected in heparin tubes. Plasma was harvested at 24 hour and 48 hour intervals. A capture enzyme linked immunosorbent assay (ELISA) was performed on the samples towards validation of the lion specific IFN-γ assay for the diagnosis of BTB. The limit of detection (109 pg/ml) and limit of quantification (850 pg/ml) of the assay were determined. A comparison was made on the difference between two incubation periods and no significant difference was found. The best mitogen for use as a positive control was determined to be phorbol 12- myristate 13-acetate (PMA)/calcium ionophore (CaI) (at 0.1 µg/ml and 2 µg/ml respectively). The diagnostic cut off for negative lions was determined preliminarily to be OD_{bov} 0.03. These preliminary results show the potential of this assay in the diagnosis of BTB as it shortens the testing turn-around time compared to the currently used intradermal tuberculin test.

Key Terms:

Bovine tuberculosis, lions, canine distemper, Interferon-gamma, ELISA, FIV, FeLV

List of Abbreviations

AEC	Animal Ethics Committee
ALERT	African Lion And Environmental Research Trust
BSA	Bovine Serum Albumin
BTB	Bovine Tuberculosis
CaI	Calcium Ionophore
CDV	Canine Distemper Virus
CMV	Canine Morbillivirus
CV	Coefficient Of Variation
CVL	Central Veterinary Laboratory
DAFF	Department Of Agriculture, Forestry And Fisheries
DG	Dangerous Goods
DNA	Deoxyribonucleic Acid
DVS	Division Of Veterinary Services
DVTD	Department Of Veterinary Tropical Diseases
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbent Assays
ELISPOT	Enzyme-Linked Immunospot
FeLV	Feline Leukemia Virus
FIV	Feline Immunodeficiency Virus
GNP	Gonarezhou National Park
HIV	Human Immunodeficiency Virus
IDT	Intradermal Test
IFAT	Immuno-Fluorescent Antibody Test
IFN-γ	Interferon-Gamma
IL	Interleukin
IUCN	International Union For Conservation Of Nature
KNP	Kruger National Park
LnIFN-γ	Lion Interferon-Gamma
LOD	Limit Of Detection
LOQ	Limit Of Quantification
LPA	Lymphocyte Proliferation Assay
MTBC	*Mycobacterium tuberculosis* Complex
NK	Natural Killer Cells
OD	Optical Density
OIE	Office International des Epizooties (World Organisation For Animal Health)
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline

PC	Peptide Cocktail
PCR	Polymerase Chain Reaction
PMA	Phorbol 12- Myristate 13-Acetate
PPD	Purified Protein Derivative
PWM	Pokeweed Mitogen
REA	Restriction Endonuclease Analysis
RFLP	Restriction Fragment Length Polymorphism
rLnIFN-γ	Recombinant Lion Interferon-Gamma
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
RT	Reverse Transcriptase
SIV	Simian Immunodeficiency Virus
TB	Tuberculosis
TGF-β	Transforming Growth Factor Beta
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumor Necrosis Factor
UK	United Kingdom
USA	United States Of America
ZVRC	Zimbabwe Veterinary Research Council

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1 LITERATURE REVIEW

1.1 Introduction

Lions (*Panthera leo*) are an iconic, culturally revered species of wildlife in Africa, which are important for tourism and biodiversity conservation by balancing animal populations through predation (Groom *et al.* 2014). Due to the massive global reduction of approximately 43% in the number of lions that have occurred in the past 21 years, lions are listed as vulnerable on the International Union for Conservation of Nature (IUCN) red list (Bauer *et al.* 2016). From an estimated 400 000 lions in the 1950s, it is estimated that the current census of lions worldwide is down to approximately 23 000 to 39 000 mature lions (Bauer *et al.* 2016).

A number of factors such as illegal hunting, increased conflict with human beings due to competition for resources as human beings encroach wildlife habitats and disease threats, some of which are due to increased human and livestock interaction have contributed to a decline in lion populations in Africa (Chardonnet(ed) 2002).

From Zimbabwe, captive lions in the country are exported regionally to countries such as South Africa, and the United States of America for tourism (ALERT 2012) for breeding purposes. Decreasing habitat, prey availability; and diseases such as feline immunodeficiency virus (FIV), canine morbillivirus (CMV) (previously known as canine distemper virus) and bovine tuberculosis (BTB), are some of the principle threats to lions in Zimbabwe (ALERT 2012).

Diseases of importance in lions are classified as either endemic (felid alphaherpesvirus 1 infection, feline leukemia virus [FeLV] and feline immunodeficiency virus [FIV], or as epidemic diseases (CMV and BTB); according to their sero-positivity rates and effects on lion populations (Caron *et al.* 2014; Ramsauer *et al.* 2007)

The disease status of most lion populations in Zimbabwe has not been documented. It is important that, should lions be brought back into the wild from a

captive state, or exported to other countries, their disease-free state should be established (Chardonnet(ed) 2002; Ramsauer *et al.* 2007). The aim of this research was to screen samples collected from three captive lion populations against three viral diseases namely FIV, FeLV and CMV for health check reasons; and perform a pilot study towards the validation of the lion interferon-gamma (LnIFN-γ) assay for BTB diagnosis in the same lion population.

1.2 Bovine tuberculosis (BTB)

BTB is a contagious, debilitating disease and during post-mortems it is characterised by the formation of nodular granulomas on affected organs (Admassu *et al.* 2015). It is considered a disease of socio-economic importance, due to its effect on animal production as result of low reproduction rates, and condemnation of milk and meat of infected animals (Admassu *et al.* 2015).

BTB can have serious effects on biodiversity conservation in two main ways. These are when the disease affects wildlife causing mortalities, and when control of the disease is aimed against wildlife maintenance hosts or reservoirs resulting in their culling (Admassu *et al.* 2015).

1.2.1 Aetiology

Mycobacterium tuberculosis and *M. bovis* are bacteria that belong to the *Mycobacterium tuberculosis* complex (MTBC), with the former causing TB in humans and the latter causing TB in cattle known as BTB. MTBC bacteria are genetically related and can cause tuberculosis in humans and other animals. *M. caprae, M. microti, M. africanum, M. canetti* and *M. pinnipedii* are examples of other bacteria belonging to the MTBC. These bacteria are aerobic, non-motile, non-spore forming rods and are termed acid-fast because they are acid and alcohol resistant; when using the Ziehl-Neelsen staining process (Ramos *et al.* 2015).

M. bovis causes TB in both domestic and wildlife species, and is an OIE-listed disease (OIE 2009).

While TB in humans is mostly caused by *M. tuberculosis*; approximately 0.2 to 7.2% of all human TB cases has been shown to be caused by *M. bovis* in developed countries (Stone *et al.* 2012). However, worldwide prevalence of TB from *M. bovis* in humans is still to be confirmed, due to the limited number of samples that have been tested in order to generalise the prevalence (Daborn *et al.* 1996). In a study carried out in West Africa in 1977, *M. bovis* was isolated from 1.8% of the human sputum samples that were tested (Daborn *et al.* 1996). In Tanzania, 8 out of 19 lymph node biopsies from TB patients were found to be positive for *M. bovis* by culture (Daborn *et al.* 1996).

In humans co-infections between the human immunodeficiency virus (HIV) and *M. tuberculosis* can result in a serious disease, due to the synergism shown by the two infections (Maas *et al.* 2012). For animals, one study by Viljoen and others (2015), proposed that co-infections of BTB with viral diseases such as FIV still need to be studied, as the pathogenicity, transmission and effect of the disease on populations are not clearly understood. In humans, co-infections between HIV and *M. tuberculosis* can result in a serious disease, due to the synergism shown by the two infections (Maas *et al.* 2012).

1.2.2 BTB in Africa

BTB is found worldwide, but is endemic in Africa. Although quantifying its public health importance is difficult in developing countries, it is known to have an impact on public health, particularly in marginalised communities of Africa (Renwick *et al*. 2007).

There has been an increasing number of wild animals reportedly affected by BTB in Africa because of increasing livestock-wildlife interface areas, intensification of surveillance and research efforts; as well as the general improvement in BTB diagnostics over the last few years (Renwick *et al*. 2007). The African buffalo is generally considered to be the main reservoir across the continent. BTB epidemiology is however known to be variable throughout Africa (Katale *et al.* 2012).

M. bovis has been isolated from the African buffalo (*Syncerus caffer*), greater kudu (*Tragelaphus strepsiceros*), lion (*Panthera leo*), eland (*Taurotragus oryx*), warthog (*Phacochoerus aethiopicus*), bushpig (*Potamochoerus porcus*), large spotted genet (*Genetta tigrina*), leopard (*Panthera pardus*), spotted hyena (*Crocuta crocuta*), cheetah (*Acinonyx jubatus*), chacma baboon (*Papio ursinus*), impala (*Aepyceros melampus*) and the honey badger (*Mellivora capensis*) (Michel *et al.* 2006).

BTB was first reported in Tanzania after the First World War (as cited in Kazwala *2001* from Hornby 1949) and has to date been found in livestock (Markham, 1952) and several species of wildlife (Cleaveland *et al*. 2005) and in human beings (Jiwa *et al*. 1997). In Tanzania, transmission from wildlife to livestock is believed to be on the rise due to human encroachment of wildlife habitats through the increasing demand for pastures and agricultural land (Katale *et al.* 2012). Its prevalence in cattle is estimated to range from 0.2% to 13.3% by area; and it has been found in 10% of apparently healthy wildebeest in one study that was carried out in the country (Katale *et al.* 2012).

BTB is believed to be either endemic or spreading in some parts of Southern Africa and many studies in wildlife have been done in Southern Africa (Renwick *et al*. 2007).

M. bovis was first described in cattle in South Africa in 1880 and is believed to have come from infected cattle imported from Europe. It was first isolated during the 1950s/60s from buffaloes in the Kruger National Park (KNP) in South Africa. Evidence suggests that the buffaloes grazed with infected cattle in the Komatipoort/Malelane area before returning to the park (Keet *et al*. 2000). The spread of BTB into the KNP led to fears of decreased biodiversity, which prompted an increase in research of the disease (Caron *et al.* 2014).

1.2.3 BTB in Zimbabwe

BTB was eradicated from cattle in Zimbabwe in 1979 through a test and slaughter campaign (Division of Veterinary Services (DVS) reports, unpublished data). Since then, reported occurrence of BTB in livestock and wildlife in Zimbabwe is scarce.

A study was carried out in Gonarezhou National Park (GNP) in south eastern Zimbabwe in 2008, where 38 African buffalo were captured and screened for BTB. Four of the buffalo were found to be positive on IFN-γ screening and culture results indicated that it was the KNP strain (de Garine-Wichatitsky *et al.* 2010). There may have been buffalo-to-buffalo transmission, as bulls and young heifers are known to constantly change herds. If transboundary movement of herds occurred and infected buffalo mixed with uninfected herds, it could have resulted in the positive result. As transboundary movement of buffalo has not been documented in these parks, a possible second species could have transmitted the disease from KNP buffalo back to GNP buffalo. The greater kudu is known to be capable of this (de Garine-Wichatitsky *et al.* 2010). Livestock could have transmitted the infection to GNP buffalo, as they commonly cross the border and transboundary movement of livestock does occur sometimes. There is also the possibility that following eradication of BTB from cattle in Zimbabwe, the presence of the disease in wildlife could have been missed (de Garine-Wichatitsky *et al.* 2010). A survey carried out following this report in domestic cattle along the GNP livestock-wildlife interface revealed a seroprevalence of 9.9 % in the cattle (Gomo *et al.* 2012). This has shown the need to carry out routine surveillance and tests for BTB in Zimbabwe.

There are ongoing efforts to test lions in the GNP. However, there are problems in capture exercises due to the vastness of the park and the relatively low populations found (approximately 33 lions in 50 000 km^2) (Chardonnet(ed) 2002). BTB is believed to be spreading northwards into the rest of the country from the GNP (Chardonnet(ed) 2002).

1.2.4 BTB in lions

Reports of BTB in lions were made from two different zoos in Germany (1992) from observations made between 1951 and 1990, and in the United States of

America (Morris & Pfeiffer 1995). The disease is said to have increased following stress, with lions exhibiting dyspnoea, poor condition and reduced body weight. It was suspected to be transmitted through ingestion of infected meat due to the intestinal lesions and through air-droplets because of the respiratory lesions observed (Viljoen *et al.* 2015).

In 1985, a male lion in a zoo in the USA also contracted *M. bovis*. Although it was in contact with three other lions in the facility these lions tested negative for BTB three years after the male lion had died (Morris & Pfeiffer 1995).

The first case of BTB in free-ranging lions was made in 1996. These were lions in the KNP (Keet *et al.* 1996). The disease was also reported in other areas like Hluhluwe-iMfolozi Park (HiP) in South Africa and the Serengeti in Tanzania (Cleaveland *et al*. 2005) where some lions tested positive when they were screened using an ELISA (Michel *et al.* 2006). There is a possibility that other lion populations around Africa are infected, due to the endemicity in livestock and buffalo, and the translocation of lions from KNP to other parts of Africa (Maas *et al.* 2013).

1.2.5 Transmission

The main route of *M. bovis* transmission, in most species, is through aerosols. In cattle however, the pathogen can also be transmitted vertically when calves ingest infected milk, and when animals graze on contaminated pastures (Bezos 2009). In rare cases congenital transmission has been reported and in very rare cases, genital transmission has also been reported (Bezos 2009).

Maintenance hosts can sustain the infection within their populations without the need for reintroduction of infection from other species. Infection in the maintenance hosts is transmitted horizontally within the herd, resulting in its persistence, even in the absence of another source (Fitzgerald & Kaneene 2013a). The possibility of wildlife reservoirs has often resulted in disease recurrence in countries with strict control programmes. Infection can therefore arise in domestic animals from contact with infected wild animals (OIE 2009).

Some species that have been shown to be maintenance hosts for *M. bovis* infection include the badger (*Meles meles*) in the United Kingdom, white-tailed deer (*Odocoileus virginianus*) in the USA, wild boar (*Sus scrofa*) in Spain and brushtail possums (*Trichosurus vulpecula*) in New Zealand (Fitzgerald & Kaneene 2013a). The African buffalo is the main maintenance host of *M. bovis* in Southern Africa (OIE 2009).

Spillover hosts are infected with *M. bovis* due to reintroduction of the infection from maintenance hosts. In these species therefore, the basic reproduction rate (R_0) or the number of new cases that can arise from one infected case is less than one (Palmer 2013). Lions are considered spill over hosts for *M. bovis* infection (de Lisle *et al.* 2002). The roles of some hosts are not defined clearly. Ferrets (*Mustela nigripes*), red deer (*Cervus elaphus*) and wild pigs (*Sus scrofa*) are regarded generally as spillover hosts (Nugent 2011), but under some circumstances such as host densities they may be considered reservoirs (Palmer 2013). Current knowledge suggests that cheetahs (*Acinonyx jubatus*), leopards (*Panthera pardus*), hyenas (*Crocuta crocuta*), large-spotted genets (*Genetta tigrina*) and warthogs (*Phacochoerus aethiopicus*) are also spillover hosts (Fitzgerald & Kaneene 2013b).

Infection in lions is believed to occur mostly through ingestion of contaminated material or infected prey (Keet *et al.* 1996). It is also believed that lions can contract the infection while suffocating their prey and during fights. Infection through the skin during exhibitions of social aggression has been cited as another possible route of transmission in lions (Viljoen *et al.* 2015).

In a study carried out by Miller and co-workers in the KNP, eight out of 134 lion samples from tracheobronchial lavage were found to be positive for *M. bovis* infection, confirming the possibility that *M. bovis* can be shed through the respiratory route (Miller *et al.* 2015).

1.2.6 Pathogenesis and immune responses to *M. bovis*

Complex interactions exist between *M. bovis* and host species, and the pathogenesis of BTB is only understood partially (Michel *et al.* 2006; Pollock *et al.* 2006). What is known about it comes partly from natural field studies carried out in cattle and from disease modelling (Pollock *et al.* 2006).

Immune responses to *M. bovis* infection are almost similar to those elicited by other non-toxin producing, intracellular bacteria (de Lisle *et al.* 2002). The cell-mediated response dominates the initial stages of infection (Waters *et al.* 2011). This response facilitates bacterial destruction by macrophages, in order to limit bacterial multiplication (de Lisle *et al.* 2002). The humoral immune response occurs later in the infection. There are usually very high concentrations of antibodies when the bacterial load increases (de Lisle *et al.* 2002).

Disease progression depends on the immune responses elicited by the host and can result in elimination of the infection, self-limitation, progression to a localised lesion or the development of a generalised systemic disease. The pathogenesis also in part depends on the route of infection transmission (Welsh *et al.* 2005).

When the pathogen is ingested or inhaled, bacterial multiplication occurs in the mucous membranes of the pharynx. Alveolar macrophages, which are antigen presenting cells (APCs), engulf the bacteria. Phagolysosomes, present in the macrophages, attempt to destroy the bacteria (Bezos 2009). The infection can be eliminated at this point if there is a low bacterial load and high host immune responses (Welsh *et al.* 2005).

If infection is not eliminated, further bacterial multiplication in alveolar macrophages can occur and the bacteria disseminated to lymphatic nodes through lymphatic vessels (Welsh *et al.* 2005). Once the lymph nodes are affected, the infection can result in a localised lesion, or a generalised infection if the infection spreads through the lymph nodes and blood into other tissues (Welsh *et al.* 2005).

Immune activation occurs in the presence or absence of phagocytosis. When mycobacteria are destroyed in phagolysosomes, this initiates activation of Interleukin R (IL1-R) associated kinase which further activates transcription factors such as nuclear factor kappa B (NF-Kb) (Bezos 2009). This stimulates cytokine production. There are three main types produced namely:

- Pro inflammatory cytokines such as
    - IFN-γ: this is a known protective cytokine especially because of its role in antigen specific T-cell immunity. It is also used as a signal for infection. IFN-γ is produced by natural killer cells (NK), lung macrophages and gamma-delta (γδ) T-cells (these form a sizeable portion of peripheral blood mononuclear cells in ruminants) (Bezos 2009).
    - Tumoral necrosis factor alpha (TNF-α): results in granuloma formation, macrophage activation and immune regulation (Bezos 2009).
    - Interleukin 1 beta (IL-1β)
    - IL-6
    - IL-12, IL-15 and IL-18: these are important for stimulating NK and T-cell proliferation and activation thereby increasing IFN-γ production
- Anti-inflammatory cytokines:
    - These affect the production or effect of pro-inflammatory cytokines and include IL-10 (antagonises IFN-γ, IL-12 and TNF α production), transforming growth factor beta (TGF-β) (reduces cell-mediated immunity and lowers IFN-γ production) and IL-4 (suppresses IFN-γ) (Bezos 2009).
- Chemokines; these attract cells to infection site and include:
    - IL-8, chemoattractant protein 1 (MCP 1) and RANTES/CCL5 (regulated in activation, normal T expressed and secreted) (Bezos 2009).

1.2.7 Clinical signs and pathology

The disease is highly asymptomatic in most species. This poses a risk when animals are moved over long distances, as they can be moved while harbouring

the infection (OIE 2009). It is therefore important to test wild animals before translocations, especially to BTB free areas, for example to zoos or to reserves (de Lisle *et al.* 2002).

Clinical signs are species dependent and some species of animals only exhibit signs in advanced disease states (de Lisle *et al.* 2002). Lesions, when present, can be found in almost any organ system, but the head and thoracic lymph nodes, lungs and pleurae are some of the organs commonly affected. Clinical signs are therefore variable and can include inappetence, emaciation, fever, enlarged lymph nodes, laboured breathing and intermittent coughing (OIE 2009). They are mostly apparent when there is disease burden or natural disasters such as droughts (de Lisle *et al.* 2002). When lymph nodes enlarge and block other systems such as the gastrointestinal tract, other signs such as diarrhoea or constipation can be found (OIE 2009).

Some clinical signs are only observed in some species and can almost be considered pathognomonic in them. These include change of behaviour in baboons and brushtail possums, which has been attributed as a possible reason for further disease spread to other individuals. In greater kudu (*Tragelaphus strepsiceros*), draining fistulae on the head nodes can occur and cheetahs can exhibit alopecia as a result of the disease (de Lisle *et al.* 2002). Buffaloes are usually asymptomatic, but can also have an arched back; on top of the general signs exhibited by most species (Katale *et al.* 2012; de Lisle *et al.* 2002).

1.2.8 Clinical signs and pathology in lions

In lions, infection with *M bovis* is characterised by elbow hygromas, wasting syndrome and eventually death (Katale *et al.* 2012). Other clinical signs shown by lions include weight loss, lameness, corneal opacity, dull coat and poor wound healing (de Lisle *et al.* 2002). Upon necropsy; gross lesions are usually found on mesenteric and peripheral lymph nodes, skin, lungs, bones and joints (de Lisle *et al.* 2002).

Lion populations without BTB have been shown to live longer than populations with the infection (Michel *et al.* 2006).

1.2.9 BTB diagnostic tests for in wildlife

Diagnostic methods for BTB in wildlife depend on whether an animal is dead or alive, the cost and feasibility depending on the species and where animals are located (Viljoen *et al.* 2015). Most disease diagnoses in wildlife are postmortal, due to sampling difficulties, getting good quality samples, field logistics and difficulties associated with identifying sick animals in the wild (Miller *et al.* 2015).

Due to the hazard group status of *M. bovis*, sterile, disposable sample containers are advocated, as environmental bacteria (potential contaminants) can grow quicker and impede the growth of *M. bovis* (OIE 2009). Secure and leak proof packaging for zoonotic organisms should be used. If testing will not be carried out immediately, refrigeration, freezing, or storage of the samples in 0.5% boric acid (bacteriostatic) for up to seven days can be done, depending on the sample (OIE 2009).

BTB is a difficult disease to diagnose based on clinical signs because of the chronic nature of the disease, as well as the relatively high percentage of subclinical infections (Ramos *et al.* 2015; Viljoen *et al.* 2015). Clinical signs such as wasting, hygromas and enlarged lymph nodes can however arouse suspicion as to the presence of the disease (Ramos *et al.* 2015).

All ante mortem diagnostic procedures available have shortcomings (de Lisle *et al.* 2002). Diagnosis in most species is based mainly on the tuberculin test and bacterial culture, with tests such as the IFN-γ assay still being developed for use in various species (Thoen 2017). Most assays pose the disadvantage of not having been validated for use in specific species; and there may be difficulties in acquiring species-specific reagents that would be needed to perform the tests (Miller *et al.* 2015).

1.2.9.1 Diagnosis at post-mortem

Post-mortem findings depend on the species and the major organs affected (de Lisle *et al.* 2002). Suspicion of BTB can be raised following necropsy findings of lymph nodes or organs with yellowish, caseous to calcified tubercles (Admassu *et al.* 2015). In lions and leopards, lesions are usually generalised (de Lisle *et al.* 2002). Lesion sizes are markedly different, ranging from almost invisible to grossly distorting an organ and whenever BTB is suspected, organs should be sectioned at multiple sites (de Lisle *et al.* 2002).

During slaughter inspections, BTB is one of the major differential diagnoses when granulomatous lesions are encountered. In species such as cattle, other disease conditions such as an *Actinomyces bovis* infection can also cause similar granulomatous lesions (Ramos *et al.* 2015). Post-mortem findings can therefore only offer a tentative diagnosis.

1.2.9.2 Histopathology

a. Microscopy

Histopathology can be used to complement post-mortem findings, in order to support a diagnosis (Ramos *et al.* 2015). Ziehl-Neelsen stained impression smears of lesions viewed under a light microscope reveal acid fast bacilli when *M. tuberculosis* complex bacteria and other mycobacterial species are present (OIE 2009). Auramine O staining, followed by fluorescence microscopy can also be used and it is believed to be more sensitive and specific than Ziehl-Neelsen staining (Schiller *et al.* 2010).

Microscopy generally has a low sensitivity, as demonstrated in studies carried out in humans. False positives can occur, as there are some species of bacteria such as *Nocardia* species that react similarly to the stain (Ramos *et al.* 2015).

In most species, lesions caused by *M. bovi*s may contain few bacilli (paucibacillary), therefore it is always advised to investigate further whenever lymph nodes are found to be grossly affected at necropsy from an unknown cause

(OIE 2009). However, bacteria can be highly concentrated at the lesion sites in primates and feline species (Schiller *et al.* 2010).

b. Cytology and serum profiles

Cytology of bronchial aspirate from BTB positive lions in one study revealed increase in macrophages, moderate mature neutrophilia, and a decrease in plasma cells and lymphocytes (Keet *et al.* 1996). Serum profiles revealed hyperglobulinemia, hypercalcemia and hypoalbuminemia; which is a similar profile as that observed for malabsorption syndrome (Keet *et al.* 1996).

1.2.9.3 Culture

Bacterial culture is the gold standard and is confirmatory for diagnosing BTB in most species (Ramos *et al.* 2015). Culture of lung samples, tracheal and bronchial lymph nodes or lavage can be carried out. The process however takes approximately 8 to 12 weeks (Wagari 2016).

For culture, tissue samples are first homogenized, then decontaminated using an acid such as sulphuric acid, an alkali such as sodium hydroxide or a detergent such as 0.375–0.75% hexadecylpyridiniumchloride (HPC) (Ramos *et al.* 2015). This is then followed by neutralization of the decontaminant, if necessary, and centrifugation. The sediment is useful for both culture and microscopy (OIE 2009). Different methods are available, but culture is carried out preferably in tightly sealed plates on both agar based medium (7H11) or egg based media such as Stonebrink supplemented with pyruvate (de Lisle *et al.* 2002).

The quality of the sample is very critical, as environmental contaminants can stop the growth of *M. bovis,* resulting in false negatives (Schiller *et al.* 2009). Decontamination of samples during the culturing process also carries the potential of destroying *Mycobacteria* activity (Ramos *et al.* 2015). It is therefore important to combine necropsy and culture for diagnosis.

Following culture, it is critical to identify and distinguish *M. bovis* from other *Mycobacteria* biochemically, or using attributes such as its optimal growth

temperature (OIE 2009). Using culture techniques for this is usually a slow, difficult and a highly inaccurate process. The need to distinguish between the different *Mycobacteria* species therefore necessitates the use of molecular techniques (Ramos *et al.* 2015).

1.2.9.4 Molecular techniques

Polymerase chain reaction (PCR) targeting different gene segments is available for the detection of *M. bovis* and its differentiation from other mycobacteria. Typing techniques such as the space oligotyping (spoligotyping) identify *M. bovis* specifically (Wagari 2016). Restriction fragment length polymorphism (RFLP) and restriction endonuclease analysis (REA) are also available to distinguish *M. bovis* from other *M. tuberculosis* complex bacteria (Fentahun & Luke 2012a). Both false positives and false negatives occur with PCR due to the paucibacillary nature of lesions thereby decreasing reliability and increasing variability of the genomic assays. These assays are therefore usually used in conjunction with culture (OIE 2009).

Olivier *et al* (2017), developed a gene expression assay for the diagnosis of lion BTB following the observation that messenger RNA assays have been developed for other species. They developed a CXCL9 gene expression assay which also makes use of one capture exercise but diagnostic cut-offs are yet to be established.

1.2.9.5 Immunology-based techniques

a. Assays based on the humoral immune response

Serology measures the humoral immune response to *M. bovis* infection (OIE 2009). The lateral flow based rapid test which is capable of detecting more than one antigen has been developed for use in elephants (Elephant TB STAT-PAK assay) for TB diagnosis (Greenwald *et al.* 2009). Serological tests using avian (Av) and bovine (Bv) purified protein derivatives (PPD) of *M. bovis* are believed to be useful for information on pathogenesis and disease progression and have the advantage of being relatively simple to use (Viljoen *et al.* 2015). They however

have a relatively low sensitivity. In a study carried out in the KNP, 12 out of 26 culture positive lion samples were seropositive for BTB (Viljoen *et al.* 2015).
Sensitivity of the assays may increase in advanced stages of the disease and when serological tests are combined with other assays that utilise the cell-mediated immune response (Viljoen *et al.* 2015). Specificity is also increased by comparing Bv PPD and Av PPD test readings (Fentahun & Luke 2012a). When there is co-infection with FIV, this might result in a reduction in antibodies in the blood stream, which decreases sensitivity of serological tests (Viljoen *et al.* 2015).

Serology is particularly useful in developing countries with poor disease surveillance and control systems as the response being tested comes later on in the disease course (Ramos *et al.* 2015). They can be used serially to increase specificity; or in parallel in order to increase sensitivity (Schiller *et al.* 2010). The major drawback of this assay is the high variation in antibody response that has been found in different animals (Fentahun & Luke 2012a).

b. Assays based on the cell-mediated immune response

1.2.9.6 Intradermal tuberculin test

The skin test is currently the standard test recommended for international trade in cattle, although it is yet to be validated for use in most species (OIE 2009). Chronically infected animals are usually non-reactors to the assay (Keet *et al.* 2010).

The test relies on the detection of delayed hypersensitivity at an injection site following injection of Bv PPD alone (single intradermal test) or in comparison (comparative intradermal test) with Av PPD at a separate injection site (Keet *et al.* 2010). The intradermal tuberculin test previously made use of *M. tuberculosis* PPD, but now makes use of the AN5 strain of *M. bovis* for the diagnosis of BTB (Ramos *et al.* 2015). Different methods exist for the interpretation of the measurements. The test sensitivity ranges between 40 to 80% and the specificity can be improved by comparison with Av PPD (OIE 2009).

The major advantage of this test is that it has been used for a long time. It is also relatively cheap to carry out depending on the species and is easily available. However, the IDT requires two visits to carry out, has a relatively poor accuracy and carries with it difficulties in standardizing and interpreting results (Ramos *et al.* 2015). *Mycobacteria* also cross react, therefore pre-exposure to environmental *Mycobacteria* can affect the test interpretation. The quality and exact quantity of PPDs used is of high importance when carrying out the IDT as it will also have a bearing on the interpretation of results (Schiller *et al.* 2010). The optimal dose of tuberculin for some wildlife species is currently unknown (de Lisle *et al.* 2002). The IDT can also be affected by the genetics of the individual being tested (Schiller *et al.* 2010). It can also be affected by confounders such as immunosuppression following capture stress, as has been demonstrated in brushtail possums and wild ferrets (de Lisle *et al.* 2002).

1.2.9.7 Lymphocyte proliferation assay (LPA)

The LPA and the IFN-γ assays detect cellular immunity (Fentahun & Luke 2012a). The LPA relies on a comparison of reactivity of peripheral white blood cells to Bv PPD and Av PPD; where the result is given as the difference between the two responses (Fentahun & Luke 2012a).

There is no need for species specific reagents when using this assay (de Lisle *et al.* 2002). It has been shown to have a higher sensitivity than serology in badgers and brushtail possums (de Lisle *et al.* 2002). Although the assay aims to increase specificity by eliminating response to other cross reacting antigens (Fentahun & Luke 2012a), it is time consuming, complex, expensive and requires trained personnel; and thus not used routinely (de Lisle *et al.* 2002). Another drawback of the assay is its use of radioactive nucleotides (Fentahun & Luke 2012b). Blood samples collected should also be tested within 24 to 30 hours following sample collection (de Lisle *et al.* 2002).

1.2.9.8 Interferon-gamma (IFN-γ) assay

The IFN-γ assay is used as another option for international trade and is often used in parallel or serially with serological tests (OIE 2009). The principle behind the assay is that antigen (Bv PPD and Av PPD) sensitised lymphocytes release the cytokine IFN-γ. This response can be measured in a capture ELISA using monoclonal antibodies against bovine IFN-γ produced by the sensitised T-lymphocytes (Ramos *et al.* 2015). The assay measures IFN-γ produced by lymphocytes following infection and is almost similar to the QuantiFERON® TB-Gold assay and has the advantage of being easy to use in the field (Olivier *et al.* 2017). The IFN-γ assay together with the IDT are useful for early cell-mediated response testing for BTB (Schiller *et al.* 2009). As the disease progresses, higher false negatives result as the cell-mediated immune response goes down (Ramos *et al.* 2015). It is useful during the early stages of infection, before infection spreads to the environment or to other animals (Fentahun & Luke 2012a). The major disadvantage of the assay is that blood should be tested within 6-8 hours after collection (Schiller *et al.* 2009).

The assay is still to be validated in some species. Currently, the Bovigam®; (Prionics) is used in cattle and has also been used in buffaloes (Michel *et al.* 2011) for the diagnosis of BTB.

1.2.9.9 TB diagnostics in lions

In lions, the main diagnostic tools for BTB include necropsy, histopathology, bacteriology and immunology. Culture is however still regarded as the gold standard (Fentahun & Luke 2012a). Samples that are normally collected for culture include lung tissue and tracheobronchial lymph nodes. Bronchoalveolar lavage can also be performed, but false negative results can occur, as aspirate fluid may not be able to reach all areas (Miller *et al.* 2015). In a study carried out in the KNP in South Africa, tracheobronchial lavage was used in 134 lions and from these; eight were found positive for BTB (Miller *et al.* 2015).

It is as unknown whether faeces, urine, saliva and mucous can be used for culture (Viljoen *et al.* 2015). Lions when affected by BTB usually have a systemic disease,

therefore it may be important to culture from all organ systems (Keet *et al.* 2010; de Lisle *et al.* 2002).

Histopathology is considered useful in lion BTB diagnosis. There is usually minimal necrosis with neutrophil infiltration on the lesions. Lesions are usually non-encapsulated and can be found focally or coalescing. There is also granulomatous inflammation with no giant cells and no mineralisation. Unlike some species; the lesions in lions have many bacterial organisms (de Lisle *et al.* 2002).

Keet *et al.* (2010) modified and validated an IDT test for lions by modifying the concentrations of reagents used. They used a dose of 0.2 ml tuberculin, double the dose used in cattle, for each injection site and only considered the reaction on the bovine tuberculin injection site during interpretation of results. The test has been validated for use in lions and approximately 86.5% of culture confirmed positives were found by the skin test (Keet *et al.* 2010). The challenge of performing this test in free-ranging lions, is recapturing the lions three days later to read the results. Not all lions will be recaptured for the readings of the results (Olivier *et al.* 2017). This exercise can also be expensive as the lions have to be tracked and immobilised when the results have to be read. The assay however has an advantage of not being affected by co-infections (Viljoen *et al.* 2015).

Feline immunodeficiency virus infection accelerates the disease progression of BTB but also leads to a decrease in the quantities of circulating antibodies thereby affecting serological test interpretation (Viljoen *et al.* 2015). ELISA or enzyme immunoassay (EIA) antibody tests using *M. bovis* and *M. avium* are also utilised in lions. Repeat sampling using these tests is believed to be useful for studying disease progression in captive lions (Viljoen *et al.* 2015). The main disadvantage of these tests is that a positive result only signifies prior exposure to infection, but not necessarily the presence of an active disease status (Viljoen *et al.* 2015).

According to Rhodes and colleagues, whole blood from feline species does not respond to various positive mitogen controls, unlike peripheral blood mononuclear cells (PBMC) (Rhodes *et al.* 2008). They studied the potential use of ELISA and ELISPOT IFN-γ assays using PBMC and found both assays to be useful in

identifying the potential presence of *M. bovis* in feline species (Rhodes *et al.* 2008).

Maas *et al*, (2010) evaluated the lion IFN-γ genetic sequence and found it to be related to the cheetah and domestic cat sequences, implying the possible usefulness of an IFN-γ assay developed for lions for BTB diagnosis in other feline species. Maas *et al*., (2012) developed a lion specific IFN-γ assay (*in vitro*) using stimulated whole blood cultures from 11 lions. Following its development, it was concluded that the assay should be optimized for use and validated by testing more lions (Maas *et al.* 2012).

The relatively low reliability of the IFN-γ assay has been shown in human beings in the presence of co-infections, as well as in the very young, suggesting that in lions, the simultaneous presence of other disease conditions such as some parasites, iron deficiency, FIV and CMV can affect the IFN-γ assay and affect its sensitivity negatively (Viljoen *et al.* 2015).

1.2.10 Control

The main burden to effective control of BTB in developing countries is that health surveillance systems in animal and human health do not have effective early warning systems. Current control methods are limited to test and slaughter policies of reactor animals to the intradermal tuberculin test as the disease takes a lot of time to treat, and treatment is not always successful (Wagari 2016).

Vaccines are however being developed for cattle and wildlife. Where present, vaccines are not being used on a regular basis because they result in difficulties in the interpretation of immunological tests (Keet *et al.* 2009).

1.3 Viral diseases

1.3.1 Feline Immunodeficiency Virus (FIV)

Feline immunodeficiency virus (FIV) is a disease of felines. Infected cats may show progressive changes in T-lymphocyte numbers, but this may not necessarily result in a poor immune system, as some cats have been found to live full lives following diagnosis with the disease (IUCN 2004).

1.3.1.1 Aetiology

FIV is a lentivirus that was discovered around 1987 and resembles the HIV. Lions are infected with lion-specific strains of FIV known as FIVple strains, for which three subtypes have been described (A, B, C) (Roelke *et al.* 2009; Troyer *et al.* 2004). Lions are believed to be infected with more than one subtype (Troyer *et al.* 2004). Throughout Africa many different FIVple strains are found, suggesting that the virus has been in the population for many years and may have become host adapted (Roelke *et al.* 2009).

1.3.1.2 Transmission and species affected

The disease affects both wild and domestic felines (IUCN 2004). FIV is mainly transmitted through bites, as the virus is highly concentrated in the saliva of cats (Hofmann-Lehmann *et al.* 1996). FIV is believed to have the potential to persist with virus shedding. Translocation of persistently infected, shedding individuals to naïve populations is dangerous as this may result in the spread of infection to other animals (ALERT 2012).

1.3.1.3 Pathogenesis and immune responses

FIV causes a depletion in T-cells with the CD4+ marker, resulting in a reduction in the CD4+/CD8+ ratio. This causes immunosuppression, resulting in immune compromised individuals with a higher susceptibility to other pathogens (O'Brien *et al.* 2012).

1.3.1.4 Epidemiology

FIV is endemic in most lion populations studied in East and Southern Africa, however no reports of FIV have been made from lions in Etosha National Park in Namibia is (Hofmann-Lehmann *et al.* 1996; O'Brien *et al.* 2012). It is believed to

be an old disease that has established itself in several wild lion populations such as those in Serengeti National Park in Tanzania (IUCN 2004).

1.3.1.5 Clinical signs

Roelke and colleagues showed evidence, through a study carried out between 1999 and 2006 in Botswana, that initial presumptions of the non-pathogenic nature of FIV in wild felids were inaccurate. Symptoms associated with HIV infection were observed in FIV positive lions. These included anaemia, gingivitis, cachexia, hepatopathy, papilloma and lymphadenopathy (O'Brien *et al.* 2012; Roelke *et al.* 2009). Mortality rates are low as a result of FIV infection, but co-infections with BTB and CMV were believed to increase mortality rates (Roelke *et al.* 2009).

FIV in domestic cats causes a reduction in white blood cells, anaemia, hyperproteinaemia and hyperglobulinemia. In lions, it has also been associated with hypergammaglobulinemia (Maas *et al.* 2012).

1.3.1.6 FIV co-infections and consequences

More often than not, infectious diseases are controlled and handled individually by health delivery services, whereas in nature, several pathogens can affect one individual at once with the possibility of synergism or antagonism in the effect (Maas *et al.* 2012).

The wasting syndrome and neurological signs associated with FIV are believed to be higher when there is co-infection with BTB or CMV (Viljoen *et al.* 2015). FIV might increase susceptibility to BTB (Viljoen *et al.* 2015). When FIVple infection co-exists with BTB, this might explain the reduced ability to identify positive animals when diagnostics are based on the cell-mediated immune response (Viljoen *et al.* 2015). However, according to a study carried out by Maas *et al* (2012), there was no evidence of synergistic effects on blood parameters between FIV/BTB co-infected lions in KNP. It could not be concluded that these effects do not exist though, as coinfection with a different strain of FIVple might have a different effect and additional stressors might also make these co-infections more important.

It was postulated that FIV contributed to the devastating CMV outbreak of 1994 in the Serengeti (O'Brien *et al.* 2012). Troyer and colleagues also showed that infection during the outbreak with FIVple A or C strains led to higher mortalities than in FIV negative lions or lions with FIVple B strain (Troyer *et al.* 2004).

1.3.1.7 Diagnosis

FIV can be suspected following signs of wasting in cats. The most commonly used methods of confirming diagnosis rely on the detection of antibodies against FIV (Bienzle *et al.* 2004). Test kits which detect antibodies against p24, which is a vital viral protein, are in common circulation. A quick field test (SNAP FIV Antibody/FeLV Antigen Combo Test; IDEXX, Westbrook, Maine, USA), and a microwell form for use in laboratories (PetChek FIV Antibody Test Kit; IDEXX) are available. Western blot can be used for FIV diagnosis. It has the advantage of being able to detect non-domestic cat antibodies but is also costly and demanding to perform. The immuno-fluorescent antibody test (IFAT) for FIV diagnosis is also available but is not routinely used as the assay's performance is not yet well known (Bienzle *et al.* 2004).

Using PCR for detecting the virus is difficult because of the variable gene sequences of FIV strains and the relatively low viral load in blood (Bienzle *et al.* 2004). Continual revisions from new diagnostic assays of what was historically presumed true is shedding more light on this disease of importance (O'Brien *et al.* 2012).

1.3.1.8 Control

As with the simian immunodeficiency virus (SIV) and HIV, the high level of genetic diversity of the virus makes it difficult to develop a vaccine. There is no cure for the disease (Troyer *et al.* 2004).

1.3.2 Feline leukaemia virus (FeLV)

Feline leukaemia virus is a highly important disease of domestic cats, and is considered an epidemic disease of lions. The virus was first described in 1964 (Alves *et al.* 2012). It may be fatal because of the anaemia, leukaemia, immunosuppression or lymphomas it causes in its hosts (Bande *et al.* 2014; Jarrett & Neil 2012). FeLV together with FIV are the two leading infectious causes of immunosuppression in domestic cats (Alves *et al.* 2012; Hofmann-Lehmann *et al.* 1996). FeLV is believed to have relatively higher morbidity and mortality rates than FIV in cats (Bande *et al.* 2014).

1.3.2.1 Aetiology

FeLV is a gamma-retrovirus which is highly unstable in the environment (Jarrett & Neil 2012). Its serotypes have variants, namely FeLV-A, FeLV-B, FeLV-C and FeLV-T. These variants are antigenically almost similar but differ in tropisms and attributes, e.g. FeLV-A is the only contagious variant (Alves *et al.* 2012). Like most retroviruses, FeLV has a high risk of undergoing genetic mutations (Hartmann & Katrin 2012).

1.3.2.2 Transmission and species affected

The disease affects feline species, particularly domestic cats (Jarrett & Neil 2012). It is rare in wildlife species with only a few published reports of confirmed positive cases of FeLV. Long term contact between cats increases the risk of transmission due to the unstable nature of the virus (Chhetri *et al.* 2015). The virus is shed in secretions and excretions and can be transmitted during grooming and when cats are sharing feeding trays. Vertical transmission of the virus is possible (Chhetri *et al.* 2015).

1.3.2.3 Pathogenesis and immune responses

The infective period of FeLV is long, as the incubation period can extend for years. The chances for genetic mutation of the virus are high during this period (Jarrett & Neil 2012). FeLV causes disease mainly by disrupting cellular immunity. Table 1.1 below; derived from Alves & dos Reis (2012) shows the different classes of disease that develop following infection:

Table 1.1: Different classes of disease and immune responses following infection
(Adapted from Alves and dos Reis, 2012; Modified from Hartmann, 2006.)

Classification of the evolution of the disease	Classification of infected animals	Immune Response	Days after infection	Healthy cat
Regressive infection extinct	Transient viraemia	Efficient virus neutralization	3-7 Days	FeLV negative-animal resistant to future infections for a period of time
Progressive	Persistent viraemia	Failure to develop an immune response	3 weeks	FeLV positive
Regressive	Latent infection	Body inactivates the virus but does not neutralize	3-13 weeks	FeLV negative (complete elimination) FeLV positive (continued viraemia)
Atypical		Complete virus is sequestered in the epithelial tissue, replicates itself but leaves the cells	3-13 weeks	FeLV positive

1.3.2.4 Epidemiology

FeLV has a worldwide distribution in domestic felids (Jarrett & Neil 2012). There are geographical variations and other factors, such as management practices and age that affect the distribution of the disease (Bande *et al.* 2014; Chhetri *et al.* 2015). Resistance to FeLV increases with age (Chhetri *et al.* 2015). While malignant lymphoma has been diagnosed in African lions, both FIV and FeLV (important causes of lymphoma in domestic felids), were not found in the populations (Harrison *et al.* 2010). Other studies also showed no FeLV seropositivity in most lion populations (Driciru *et al.* 2006).

1.3.2.5 Clinical and post mortem signs

The resultant clinical signs are highly variable, but are mostly due to the immunosuppression caused by the virus. The disease can cause haematopoietic tumours and disorders and opportunistic infections of bacterial, fungal, viral and protozoal origin (Alves *et al.* 2012). Immune mediated disorders, reproductive disorders and the 'fading kitten syndrome' characterized by wasting can be observed (Hartmann & Katrin 2012).

1.3.2.6 Diagnosis

Clinical signs are usually suggestive of the presence of infection. However, clinical diagnosis is usually based on the demonstration of the FeLV-p27 antigen; which is the major core protein during peak viraemic states (Alves *et al.* 2012; Bande *et al.* 2014). This is usually done using the direct immunofluorescence test and ELISA (Alves *et al.* 2012). Early and late viraemic states require the detection of viral genetic material, namely viral RNA and proviral DNA respectively (Bande *et al.* 2014). Viral isolation is not routinely carried out for FeLV diagnosis (Alves *et al.* 2012).

1.3.2.7 Control

The major control strategies employed in domestic cats include separating infected cats from non-infected cats. In recent years, vaccination has also been widely used to control the disease (Jarrett & Neil 2012). Antiretroviral drugs are sometimes used in domestic cats to slow down disease progression (Alves *et al.* 2012).

1.3.3 Canine morbillivirus (CMV)

It is postulated that epidemic viruses have no consistent behaviour and have unpredictable effects on the health status of lions, but CMV has so far been the most devastating disease on lion health and numbers (Packer *et al.* 1999). A CMV outbreak in the black-footed ferret (*Mustela nigripes*) resulted in its extinction in the wild (ALERT 2012). Due to the continued increase in human population, and resultant increase in dog populations, which were believed to have been the reservoir for the 1994 Serengeti outbreak, there is fear that more CMV outbreaks might occur in the future (Packer *et al.* 1999).

1.3.3.1 Aetiology

CMV is an enveloped RNA virus (genus Morbillivirus, family *Paramyxoviridae*) with a worldwide distribution and is structurally similar to the rinderpest and measles viruses (Viana *et al.* 2014). It is unstable in the environment due to its enveloped nature (Guiserix *et al.* 2007).

1.3.3.2 Transmission and species affected

CMV is contagious and is known to be transmitted through aerosols (Guiserix *et al.* 2007). It affects a wide range of species including both wild and domestic canids, felids, marine mammals and non-human primates; with a high potential for switching between hosts (Viana *et al.* 2014). Domestic dogs (*Canis lupus familiaris*) and other canines are believed to maintain the infection (Viana *et al.* 2014).

CMV affects both wild and captive lions, with the potential to devastate and markedly reduce populations. Infection in large populations can be maintained, but it usually disappears from smaller ones until re-introduction occurs from external sources (Viana *et al.* 2014). During the 1994 outbreak, which was believed to have come from domestic dogs, all lions in the Serengeti were believed to have been susceptible (Hofmann-Lehmann *et al.* 1996), resulting in rapid spread of the disease and high mortality rates which killed approximately 30% of the total lion population in the park.

1.3.3.3 Pathogenesis and immune responses

CMV has an incubation period of three to seven days (Guiserix *et al.* 2007). The virus initially infects macrophages and lymphoid cells of the upper respiratory tract; then spreads to other body organs resulting in either subclinical or clinical infection (respiratory, digestive or neurological signs) (Jóźwik & Frymus 2005). CMV is known to have high immunosuppressive effects in their hosts (Guiserix *et al.* 2007). Following the appearance of clinical signs, viral excretion occurs over a period of a fortnight to one month (Guiserix *et al.* 2007). This culminates in either death or recovery of an animal with immunity from the disease thereafter (Jóźwik & Frymus 2005).

1.3.3.4 Epidemiology

The disease has a worldwide distribution (Guiserix *et al.* 2007). CMV is known to have a high prevalence in domestic dogs in southern Africa, but there have been few reports of its occurrence in lions in the region (Ramsauer *et al.* 2007). Lions in southern Africa are presumed to have a lower susceptibility to epidemic viruses, due to the density dependent nature of viral disease outbreaks (Ramsauer *et al.*

2007). However, CMV exhibits host cross-over properties that make it a dangerous pathogen, particularly for endangered species (Viana *et al.* 2014). In a study carried out in the Serengeti Park, CMV and other viruses had varied sero-positivity rates, and epidemics coincided with the introduction of susceptible lions into the population (Packer *et al.* 1999).

1.3.3.5 Clinical and post mortem signs

Signs and symptoms of disease in lions are almost similar to those observed in domestic dogs (*Canis familiaris*) (Guiserix *et al.* 2007). These include vomiting, diarrhoea, purulent discharges, pneumonia, seizures, ataxia, myoclonus and encephalitis (Carpenter *et al.* 1998; Dybas 2009; Guiserix *et al.* 2007). High mortalities have been attributed to concurrent immunosuppression, for example with FIV, climate changes and repeated introduction of the virus into populations (ALERT 2012; Guiserix *et al.* 2007).

1.3.3.6 Diagnosis

The direct immunofluorescence assay is widely used for CMV diagnosis (Jóźwik & Frymus 2005). Samples such as nasal smears are incubated with mono or polyclonal antibodies against CMV, which are marked with fluorescein (Jóźwik & Frymus 2005). The test however has a high potential for false negatives in chronic cases as the virus disappears from circulation after a relatively short period of time (Jóźwik & Frymus 2005).

Serum neutralization tests (SNTs), ELISA and IFAT have been used, but only indicate prior exposure and is therefore not useful in cases where vaccination is carried out (Jóźwik & Frymus 2005). Reverse transcription PCR, virus isolation and identification are confirmatory of disease presence (Jóźwik & Frymus 2005).

1.3.3.7 Control

The major strategy used for the control of CMV in domestic dogs is through vaccination that is carried out while dogs are still young, depending on the vaccination protocol used in a country (Jóźwik & Frymus 2005). There is no treatment for the disease and following infection, most cases end in fatalities.

1.4 Problem Statement

The global population of lions is on the decline and diseases affecting lions are among the causes for this decline. The status of most Zimbabwean captive lion populations with regards to both endemic and epidemic diseases is not known. This is because of the lack of human, financial and material resources for carrying out capture exercises and routinely screening for diseases. There is documented evidence of the presence of BTB in livestock and wildlife populations in Zimbabwe. BTB is a major threat due to its transboundary nature, and for public health concerns due to its zoonotic nature. Viral diseases such as FIV, CMV and FeLV are also important as they have the ability to reduce lion populations by their resultant morbidities and mortalities.

1.5 Justification of the Research

All lions for translocations, particularly in zoos, require screening for a panel of tests, including BTB. Recapturing for testing using the widely used skin test is not only expensive, but requires well trained personnel for immobilization, performing the test and reading the result. The *in vitro* IFN-γ assay, which only requires one capture was developed for use in lions by Maas *et al*., (2012) and optimised using samples collected from lions in the KNP (Khumalo 2017). The LnIFN-γ assay has not yet been validated for use in lions.

1.6 Aims and Objectives of the Study

The aim of this study was to perform a pilot study towards validating the IFN-γ assay for use in the diagnosis of BTB in captive lion populations in Zimbabwe, while screening for CMV, FeLV and FIV in the same populations.

1.7 Study Objectives

1. To collect, process and test samples with the newly optimised lion IFN-γ assay, as a step towards validation of the test.

2. To determine the diagnostic cut-off for BTB negative lions from the IFN-γ assay.
3. To determine the seroprevalence of FIV, FeLV and CMV in three captive lion populations in Harare, Zimbabwe.

1.8 Value of the Research

The research is a small contribution towards the disease status of three captive lion populations in Zimbabwe, which would otherwise not be known due to the resource constraints that inhibit this exercise to be performed on a regular basis. The results and conclusions drawn from this study will be useful as a first step towards the validation of the LnIFN-γ assay for BTB diagnosis. Furthermore, this research is a contribution towards attaining a Master of Science (MSc) degree.

1.9 Ethical and Biosafety Considerations

Ethical approval for the project was obtained from the Animal Ethics Committee (AEC) of the University of Pretoria, Faculty of Veterinary Science (approval number v055/16) and from the Zimbabwe Veterinary Research Committee (ZVRC) (see appendix). As per the requirement of the AEC and the ZVRC, consent from the various park owners was also obtained before the samples were collected from the animals; prior to the committees approving the project.

A Section 20 application was also obtained for the study from the South African Department of Agriculture Fisheries and Forestry (DAFF) (#201610000699). Since the samples were tested in South Africa, they had to be imported into the country from Zimbabwe. For this, a veterinary import permit for the importation of plasma and serum samples was obtained from DAFF (veterinary import permit number: 13/1/1/30/2/0-20161000699) (see appendix).

2 MATERIALS AND METHODS

2.1 Introduction

All the work that is outlined in this research was carried out during the research period. This includes lion captures for sample collection, sample processing and preservation, sample testing and data analysis.

2.2 Sample collection areas and sampling population

Fifteen lions (*Panthera leo*) were selected randomly from three wildlife parks (Parks A, B and C) within a 50 km radius of Harare. The lions were handled within their natural habitat. No lions were taken out of the parks to other facilities during the study. The 15 lions were aged between 3.5 to 20 years. Only one lion from Park C was born and brought in from the wild. The rest of the lions were born in captivity.

The disease status of these lions for BTB and viral diseases was unknown prior to this study. There was no change in the management of the lions before and during the study.

From Park A, which housed 27 lions, seven lions (two females and five males) were sampled. Three of these animals were brought in from other parks. The lions are fed mostly with horse meat or beef from cattle mortalities from surrounding municipal farms. They are also fed on beef from condemned carcases from abattoirs.

Park B is a sanctuary for various wildlife species including cheetahs, giraffes, wildebeest and eland. The park had a total lion population of nine, from which four were sampled, specifically two males and two females. All four lions were brought in from another park in Harare. Both male lions were vasectomised and the females had contraceptive implants as it was not intended for them to breed. The lions were fed on donated horse meat.

Park C is predominantly a lion and bird sanctuary. It had a total lion population of 13 from which four (two females and two males) were sampled. All the animals in this

sanctuary were brought in from different places in Zimbabwe and one of the sampled lions was born in the wild. The lions are fed on chicken, pork and beef from animal mortalities donated from farms around Harare. All lions are housed in pens demarcated by fences.

Table 2.1 below shows the data on the lions that were sampled for the project. The Table shows the ages, sexes and the relevant history of where each of the sampled lions came from.

Table 2.1: Lions sampled for the project

Park	Name of lion	Sex	Age (yrs)	Lion history	Vaccination history
A	Lilo	F	9	Born on site	Rabies
A	Lola (white lion)	F	5	Free State, South Africa	Rabies
A	Stitch	M	9	Born on site	Rabies
A	Hombarume	M	7	Karoi, Zimbabwe	Rabies
A	Tsotsi	M	12	Chegutu, Zimbabwe	Rabies
A	Temba	M	16	Born on site	Rabies
A	Whiskey	M	16-17	Born on site	Rabies
B	Grand	M	3.5	From park in Harare	Rabies
B	Nyasha	F	3.5	From park in Harare	Rabies
B	Diana	F	7.5	From park in Harare	Rabies
B	Joe	M	7.5	From park in Harare	Rabies
C	Ma'wire'	M	15-20	Brought in from wild	Rabies
C	Joshua	M	15-20	Masvingo, Zimbabwe	Rabies
C	Johanna	F	10	Brought in	Rabies
C	Juno	F	15	Brought in	Rabies

2.3 Clinical examination

A lion physical examination form (see appendix) (supplied by Skukuza Veterinary Wildlife Services, South Africa) was used as a guideline to carry out physical examinations in all the lions. The approximate body condition score (0 to 5), weight, size of lymph nodes, presence or absence of wounds, ocular lesions, skin condition, gingivitis, papillomas and presence or absence of ticks were some of the

observations noted for each animal. The pulse and respiratory rates were monitored during the anaesthesia in order to establish the depth of anaesthesia.

2.4 Sample Collection

2.4.1 Lion capture

A state registered wildlife veterinarian carried out the lion immobilizations and was responsible for monitoring them during and immediately following anaesthesia. Carbon dioxide propelled pneudarts (Global supplies) from a DANiNJECT® dart gun were used to dart all the lions used in this study. Lions were immobilised using 3-5 mg/kg tiletamine/zolazepam (Zoletil, Virbac) combined with 0.03-0.05 mg/kg medetomidine (Kyron laboratories Pvt Ltd). Reversal was performed using atipemazole (Antisedan®; Kyron laboratories Pvt Ltd) at a dosage five times the medetomidine dose.

Following darting, the lions were observed for effects of anaesthesia and for signs of distress. Approximately 10 to 20 minutes following darting, depending on the time to effect for each individual animal, the lions were moved by park personnel into handling pens where they were placed in lateral recumbence; and the eyes were blindfolded using a clean wet cloth. The tongues were extruded in order to monitor any colour changes during anaesthesia. Darting and anaesthesia reversal times were noted down for each lion.

2.4.2 Collection of blood samples

Approximately 10 to 15 ml of blood was collected in two 9 ml heparin vacutainer tubes, while approximately 5 ml of blood was collected into one 9 ml serum vacutainer tubes from each lion. Details pertaining to sample collection, which included lion identity, sample area, the age and sex of the lion, and time of sampling, were recorded. The blood samples were transported to the laboratory in a polystyrene box at ambient temperature (20 to 25°C) (heparin tubes) and in an upright position on ice for the serum collection tubes.

2.4.3 Transportation and storage of samples

A company equipped to transport the samples from Zimbabwe to South Africa was used. The samples were packaged for transportation using triple layer packaging. The inner receptacle constituted of the Axygen tubes, then plastic sealed boxes sealed using parafilm. Dangerous goods (DG) boxes were used as the outer receptacle. Both the plasma and serum samples were transported from the Central Veterinary Laboratories (CVL) in Harare, Zimbabwe to the Department of Veterinary Tropical Diseases (DVTD), Onderstepoort, South Africa, by air freight. Samples were transported on dry ice from their point of collection (CVL) until their delivery to the DVTD where they were stored immediately at -80 °C.

2.5 Lion IFN-γ ELISA

2.5.1 Whole blood processing

All blood samples collected in heparin tubes were processed at the CVL in Harare within four to six hours following collection. The blood was diluted 1:1 with RPMI medium (Sigma, Cat. No. RB759-1L), divided in 1 ml volumes into a 24-well tissue culture plates and stimulated using one of the following conditions: bovine purified protein derivative (BvPPD, Prionics, Cat. No. 7600060) (20 µg/ml), avian PPD (20 µg/ml) (Prionics, Cat. No. 7600065), phorbol 12-myristate 13-acetate (PMA)/calcium ionophore (CaI) (0.1 µg/ml and 2 µg/ml respectively) (Sigma, PMA, Cat. No. P8139-1MG and CaI, Cat. No. C7522-5MG), pokeweed mitogen (PWM) (640 µg/ml) (Prionics, Cat. No. 5108777) or peptide cocktail (PC) (Prionics, Cat. No. 7600100) (3 mL (lyophilized), concentration 1 mg/ml), RPMI 1640 medium (Sigma). Blood stimulated with PMA/CaI and PWM served as the positive controls for cell competency. Blood stimulated with medium only served as the negative control.

The blood was incubated at 37°C for 24 and 48 hours. The plasma was harvested after the respective incubation periods into labelled duplicate Axygen 1.1 ml tubes (SG, AX/MTS/-11-CR-S/S). The supernatant was stored at -80°C until use in the ELISA.

2.5.2 Lion IFN-γ Capture ELISA

The capture ELISA was carried out according to the procedure described by Maas *et al*, (2012) with some modifications. A 1% bovine serum albumin (HYCLONE BSA) (Separations, Cat. No. SH30574.02) in phosphate buffered saline (PBS) preparation was used as blocking buffer and diluent, instead of 1.3% universal casein diluent. Greiner Bio One microlon (extra high binding, 655061, E120700P). Plates were coated with 50 µl capture antibody (MoAb Li2B7.2G7) at a concentration of 2 µg/ml. The plate was then incubated for one hour in a shaker at 250 rpm at ambient temperature. The coating was discarded and the plate tapped dry. All subsequent incubation steps, except for the substrate step, were performed at ambient temperature, shaking at 250 rpm. Blocking was performed using 200 µl of 1% BSA in PBS and the plate was incubated for 15 min. The blocking buffer was then discarded and the plate tapped dry. The plate was washed three times with 0.1% Tween-20 (Sigma-Aldrich, Cat. No. P1379-250ML) in Biochrom PBS solution (SG, Cat. No. BC/L 1835- 500ML) using an ELISA plate washer (PW40 BioRAD ELISA washer) and tapped dry. Into the appropriate wells, as highlighted by the plate layout exemplified in the figure below, 50 µl of the samples were then added leaving two columns for the addition of recombinant lion IFN-γ (rLnIFN-γ).

The example in Figure 2.1 below shows the layout that was used for the standard curve on the ELISA plates.

	Standard curve											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	STD1 7000	STD1 7000										
B	STD2 3500	STD2 3500										
C	STD3 1750	STD3 1750										
D	STD4 875	STD4 875										
E	STD5 437.5	STD5 437.5										
F	STD6 218.75	STD6 218.75										
G	STD7 109.38	STD7 109.38										
H	STD8 0	STD8 0										

Figure 2.1: ELISA plate layout for standard curves.

For the standard curve preparation, rLnIFN-γ was used at a starting concentration of 7000 pg/ml which was serially diluted to a final concentration of 54 pg/ml. No rLnIFN-γ was added to the final well and served as the blank. A clean, separate, bacteriology grade plate was used to prepare the standard curves. From the dilutions, 50 µl from each well was transferred into the appropriate well on the ELISA plate. The plate was incubated for 1 hour, followed by the wash step as described previously. Following this, 50 µl of the detecting antibody (MoAb Li7A9B4), at a dilution of 1:5000, was added to the plate. The plate was incubated for 30 min, then washed three times. The streptavidin conjugated to horseradish peroxidase (streptavidin-HRP80 conjugate) (Streptavidin-Peroxidase Polymer Ultrasensitive cat number S2438) was used at a 1:1000 dilution (Khumalo 2017) and 50 µl was pipetted into each well. The plate was incubated for 15 min, after which it was washed six times. The substrate, 50 µl TMB (Pierce), was added into each well. The plate was incubated for 15 min and the reaction stopped with stop solution (50 µl sulphuric acid-2 M H_2SO_4 per well). An ELISA plate reader (Powerwave, BioTek) was

used to read the plates, using an appropriate protocol. The ELISA plates were read at OD_{450} (test) and OD_{620} nm (blank read). The final reading was computed as OD_{450} - OD_{650}. The concentration of the samples was determined using the four parameter logistic regression (4PL).

2.6 Serological evaluation of viral disease status

Serum was harvested from the plain tubes after 24 hours following refrigeration at 4 to 8°C. Two aliquots of serum per animal were harvested into labelled 1.5 ml tubes and stored at -80°C.

Serum samples were thawed and vortexed prior to testing. Immediately after thawing each sample, the manufacturer's instructions were followed in performing each assay (see appendix). Observations from each assay were recorded into a spreadsheet.

2.6.1 Anigen Rapid FIV Ab/FeLV Ag test kit® principle

The Anigen Rapid FIV Ab/FeLV Ag test kit® (Bionote, Inc) was used to screen for FIV and FeLV. The assay is chromatographic and dependent on the observation of a purple line on both the test (T) and control (C) lines written on the surface of each test device. The control line should be present in both negative and positive samples in order to ascertain proper performance of the assay. This is a qualitative immunoassay for the detection of FIV antibodies and FeLV antigen in feline species. The assay contains FIV antigens which capture and detect FIV antibodies should they be present in serum, plasma or whole blood. It also contains FeLV antibodies which capture and detect the presence of FeLV antigens in feline samples.

Three drops of the sample were placed into the assay diluent and mixed. The mixture was incubated at room temperature for one min. A dropper was then used to take this mixture of the sample and assay diluent and place four drops into the sample hole. The results were read after 10 min. The procedure was repeated for all 15 lion samples using a new test kit for each sample.

2.6.2 Anigen Rapid CDV Ag Test Kit® principle

The Anigen Rapid CDV Ag Test Kit® (Bionote, Inc) was used to screen for CMV. The assay relies on the observation of a purple line on both the test (T) and control (C) lines written on the surface of each test device. The control line is a reference and should also be present in both negative and positive samples in order to ensure that the assay is performing properly. It is also a qualitative immunoassay used for the detection of CMV antigen in canine species with high accuracy. The assay contains CMV antibodies which are used to capture and detect antigens if they are present in serum, plasma, conjunctiva or urine. Using a capillary tube, one drop of a sample was placed into two sample holes (FIV and FeLV). Two drops of the assay diluent were then added into each sample hole. The results were observed and recorded after 10 min. The procedure was repeated for all 15 lion samples using a new test kit for each sample.

2.7 Data Analysis

Data for clinical examination that was captured on the physical examination forms and the serology results was captured into Microsoft Excel spreadsheets and results were presented in a Table.

Samples were tested in duplicate in the LnIFN-γ ELISA. The final OD values were computed as an average of the difference between the test and the blank readings; i.e OD_{450}-OD_{652}. The mean, standard deviation and the coefficient of variation were calculated in Microsoft Excel.

RPMI 1640 media was added to blood samples during the stimulation phase to act as the negative control, while PMA/CaI was used to stimulate sample and act as the positive control. These were included to assess the cells' competency to respond to stimulation. No samples were excluded from the analysis based on the negative and positive control results.

Statistical analysis for comparing the differences between incubation time and mitogen stimulation was performed using the two tailed Student t-test in Microsoft Excel.

The lower limit of detection of the assay was calculated as three times the OD_{450} reading of the negative control after removing all readings that had a coefficient of variation (CV) 20% and higher. The limit of quantification was calculated as ten times the OD_{450} reading of the negative control after removing all readings that had a coefficient of variation (CV) 20% and higher.

The diagnostic cut off for BTB negative lions was calculated using considerations provided for in the OIE Manual of diagnostic tests and vaccines for terrestrial animals, (2017) as well as the calculations used by Morar *et al,* (2013) towards establishing a rhinoceros-specific IFN assay for BTB diagnosis. The following equation was used in the study:

$$\mu\ (OD_{bov\ -\ nil}) + 2{*}\delta_{standard\ deviation}\ (OD_{bov\ -\ nil})$$

3 RESULTS

3.1 Sample collection, processing, and data recording

3.1.1 Clinical examination

A lion physical examination form was used as a guideline to carry out physical examinations in all the lions. The animals were all healthy, with none showing signs of obvious systemic infection or disease upon clinical examination. None of the lions had hygromas, ulcerated skin lesions, enlarged lymph nodes, periostitis or osteomyelitis. All the lions scored as normal for FIV. However, six of the seven lions from Park A were infested with ticks, while only two of the four lions from Park B had ticks on the coat. None of the lions from Park C had a tick infestation.

One lion from Park B was partially blind and was reported to have been like this since birth. One lion from Park C was overweight and was reported to have been brought-in, in an over conditioned state.

3.2 Serological evaluation of viral disease status

After following instructions from the manufacturer on the test procedure, observations were made and recorded onto a data collection sheet. All test kits showed a single control line developing after the test allotted time. No colour developed along the test line. This indicated that all 15 lions tested serologically negative for CMV, FeLV and FIV.

3.3 Lion interferon-gamma (LnIFN-γ) ELISA

3.3.1 IFN-γ monoclonal capture antibody

Results of the titration curve for rLnIFN-γ using a coating concentration of 2 µg/ml for the capture monoclonal antibody is shown in Figure 3.1 below.

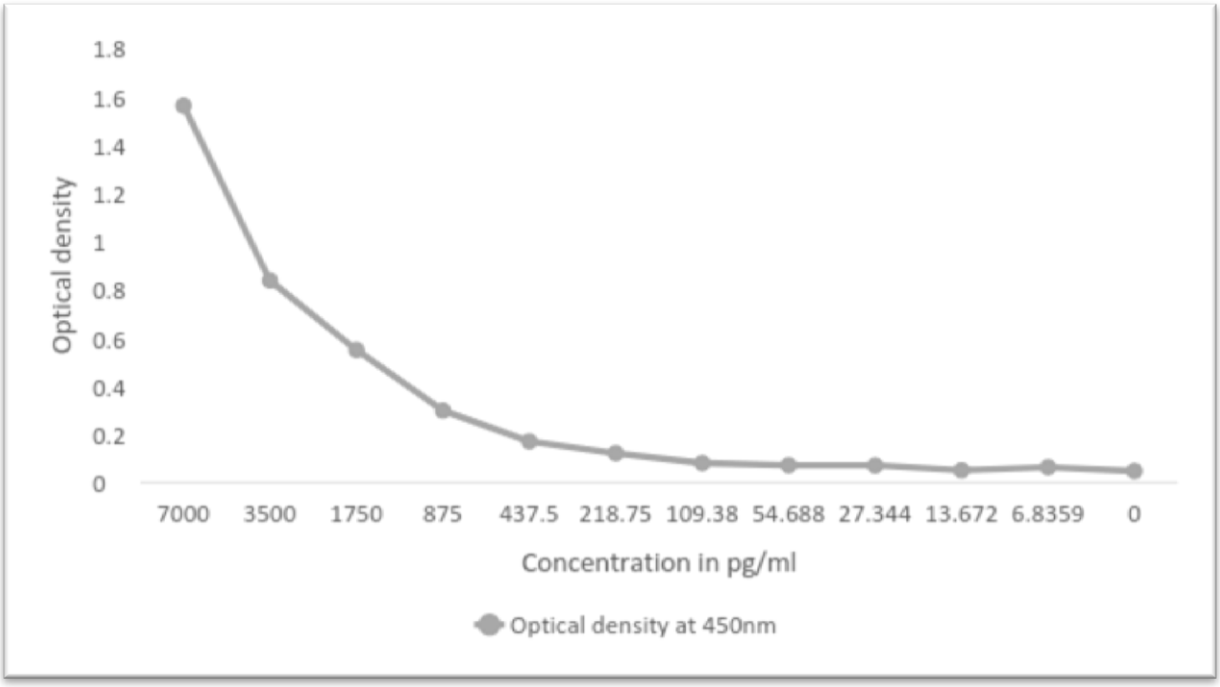

Figure 3.1: Titration curves for recombinant lion interferon- gamma (rLnIFN-γ). The limit of detection (LOD) was determined to be 109 pg/ml and the limit of quantification (LOQ) was found to be 875 pg/ml.

3.3.2 Mitogen stimulation

The results of the mitogen stimulated samples (n=12) gave different IFN-γ concentrations, with PMA/CaI giving consistently high readings at both 24 hour and 48 hour incubation intervals.

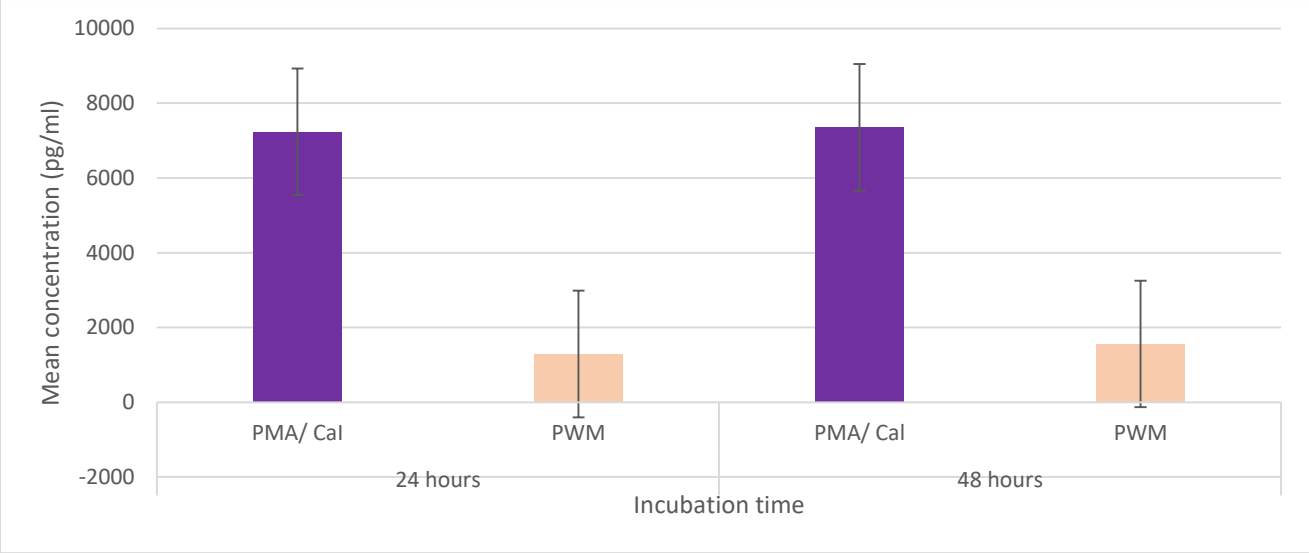

Figure 3.2: Comparison between stimulating with phorbol 12-myristate 13-acetate (PMA)/calcium ionophore (CaI), pokeweed mitogen and PWM (n=12). OD values for blood samples which were not stimulated (negative/nil controls) were subtracted from the mitogen stimulated samples to obtain the final values.

A comparison between mitogen stimulation at OD_{450} between the 24 hour and 48 hour incubation periods was made. The results are shown in Table 3.1 below.

Table 3.1: Comparison between mitogen stimulation at OD 450 between 24 and 48 hour incubation periods for n=12.

	24 hrs		48 hrs	
	PMA/CaI	**PWM**	**PMA/CaI**	**PWM**
Mean (pg/ml)	7229.78	1292.47	7350.00	1561.64
Standard deviation	317.03	930.28	0.00	987.87
p (24h vs 48h)	0.18	0.50	0.18	0.50
p (PMA vs PWM)	2.56E-17		3.87E-16	

From the given p values for each mitogen between the two incubation times, ($p > 0.05$), there was no significant difference in responses between the two incubation periods. There was however a significant difference ($p < 0.05$) between stimulation with the different mitogens (PWM vs PMA/CaI). No samples were excluded from the analysis based on the results from the PMA/CaI values.

3.3.3 Comparison between whole blood stimulation with bovine PPD and avian PPD at 24h and 48h incubation times

An evaluation of the difference between the harvesting concentrations of the two antigens used in this study (bovine PPD and avian PPD) was carried out. The results are shown in Figure 3.3 below.

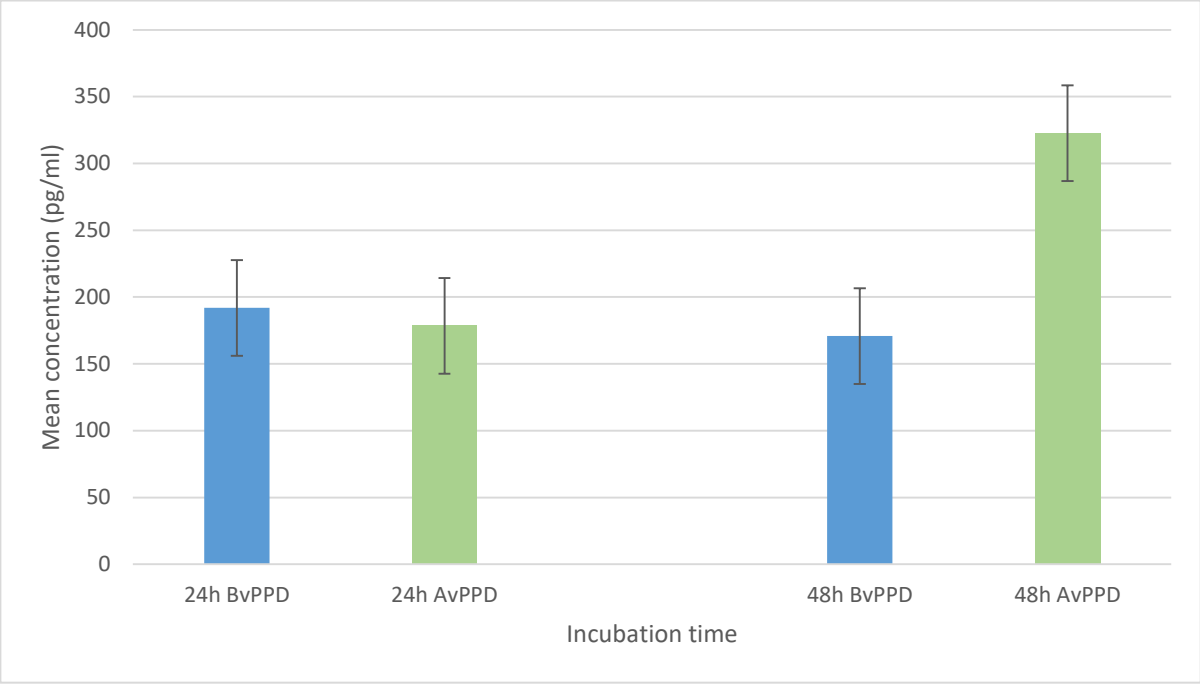

Figure 3.3: Comparison between bovine protein purified derivative (BvPPD) and avian purified protein derivative (AvPPD) after 24 and 48 hours incubation for n=13.

The mean concentrations of IFN-γ produced for the 24 and 48 hour incubation by Bv PPD stimulation were almost similar whereas AvPPD recorded higher concentrations for the 48 hour harvesting than the 24 hour incubation time. This may have been a result of readings from two lions (LKV1 and LKV15), which recorded higher concentrations than the rest of the samples for this incubation period. The mean concentrations for Av PPD were 198 pg/ml and 251 pg/ml for the 24 hour and 48 hour incubation period respectively. The mean concentrations for Bv PPD were 232 pg/ml and 202 pg/ml for the respective incubation times. Standard error bars are shown in the above mentioned figure.

No significant IFN-γ (ng/ml) production was found after antigenic stimulation with bovine and avian PPDs, as well as peptide cocktail; in comparison with the negative control. Responses at OD_{450} to Bv PPD stimulation ranged from 0 to 0.017 after nil

subtraction while those to avian PPD stimulation ranged from 0 to 0.025 after subtraction of nil values.

Table 3.2 below shows a comparison between Bv PPD and Av PPD stimulation at OD_{450} between the 24 hour and 48 hour plasma harvesting.

Table 3.2: Comparison between Bv and Av PPD stimulation between the 24 hour and 48 hour incubation intervals

	24 hrs		**48 hrs**	
Antigen	**Bv PPD**	**Av PPD**	**Bv PPD**	**Av PPD**
Mean (pg/ml)	191.92	178.45	170.84	322.56
Standard deviation	165.19	145.78	88.71	293.69
p (24h vs 48h)	0.71	0.16	0.71	0.16
p (Bv PPD vs Av PPD)	0.84		0.12	

From the given p values, ($p > 0.05$); there was no significant difference in responses between the 24 hour and 48 hour incubation intervals. There was also no significant difference between stimulation with the Bv and Av purified protein derivatives.

3.3.4 Diagnostic cut-off for negative lions

The diagnostic cut off value for a negative test for lions was calculated according to the given formula and found to be 0.03 at an $OD_{450\ nm}$. According to this cut off value, no lions showed elevated bovine PPD stimulations.

4 DISCUSSION

In this research project, a pilot study towards validating the IFN-γ assay for use in the diagnosis of BTB in captive lion populations in Zimbabwe was performed, while screening for CMV, FeLV and FIV. The results from this study serve as an initial communication towards validating the IFN-γ assay for use in the diagnosis of BTB, as well as highlighting the health status of the lions with regards to the diseases highlighted.

Disease diagnosis in wildlife is often carried out at the post-mortem stage, particularly in free-ranging wildlife. It is however important to make efforts to carry out ante-mortem diagnosis for biodiversity conservation, translocation and for public health reasons. The diagnosis of BTB is in itself complex, but becomes more challenging in wild animals (de Lisle *et al.* 2002). Several factors are responsible for this complexity including the difficulties and expenses incurred during capturing wild animals. Subclinical infections also complicate the diagnosis of BTB (de Lisle *et al.* 2002). The development and validation of an assay that would make this diagnosis easier in one way or another is critical for determining the health status of free-ranging animals.

Previous work carried out by Maas *et al.* (2012) was aimed at developing the LnIFN-γ assay for diagnosis of BTB in lions. In a study carried out by Khumalo, (2017) the optimum concentrations of the capture and detecting antibodies, the conjugate the blocking buffer were determined. In Khumalo's study (2017), 1% BSA in PBS was used as the buffer in place of 1.3% universal casein (Maas *et al.* 2012). This project used recommendations for optimal concentrations from Khumalo's study in order to determine the limit of detection as well as limit of quantification of the assay. More samples were evaluated using the assay. In addition, all samples were screened against three immunosuppressive infections namely FIV, FeLV and CMV.

Samples were collected successfully from all fifteen lions that had been planned for. However, not all samples were used for the analysis due to the coefficient of variance cut off that had been set at 20%. An average of approximately 12 samples was used for each analysis. None of the animals had been exposed to *M. bovis* or were suspected to have come into contact with *M. bovis*.

Mitogens were used in the study to evaluate the best positive control when stimulating whole blood cells. Samples from all animals were stimulated using PMA/CaI and PWM. PMA/CaI had the best responses due to the consistently high OD values achieved in all samples. This is in line with the recommendation to use PMA/CaI as the cell viability control by Maas *et al*., 2012 in the development of this assay and (Khumalo 2017) in optimizing the assay. A previous study carried out in feline species has shown that whole blood does not respond to various positive mitogen controls, unlike peripheral blood mononuclear cells (Rhodes *et al.* 2008).

Bovine and avian PPDs as well as peptide cocktail consisting of ESAT-6 and CFP-10 antigens were used to stimulate whole blood cells to detect prior exposure of *M. bovis* in lions. The use of these antigens is suggested to increase the specificity of the assay without compromising its sensitivity (de Lisle *et al.* 2002). Relatively low reactions were observed for all three antigens. This suggests that none of the lions stimulated had previous exposure to the major *M. tuberculosis* complex antigens which correlated to the history and physical examinations carried out on the lions.

After 48 hours incubation, OD_{450} of bovine stimulated blood had dropped and increased for avian PPD stimulated blood for two lions. This observation could be due to environmental mycobacterial species which can increase the avian PPD stimulated blood OD_{450} results (Fentahun & Luke 2012).

There was no significant difference between harvesting the plasma at 24 hours and at 48 hours in samples that were evaluated. This shows the potential of the assay to have a quicker turn- around time than standardized assays such as the intradermal test.

Maas *et al*. (2012) used es(HS) TMB reagent (SDT, Germany) and the colour reaction was stopped after 10 minutes. The TMB (Pierce) used in this study had minimal colour reaction after 10 minutes, therefore stoppage time for the reaction using sulphuric acid was increased to 15 minutes. In the optimization study (TMB substrate, Sigma, T4444), the reaction was stopped after 20 minutes. This shows that stoppage time when carrying out the capture ELISA may depend on the TMB being used.

The detecting antibody and conjugate was used at a dilution of 1:5 000 according to recommendations from a previous study (Khumalo 2017). The lower limit of detection for rLnIFN-γ was found to at 109 pg/ml. This LOD was in line with what was determined in two previous studies which could detect at 109 pg/ml (Khumalo 2017) and 160 pg/ml that was previously described by Maas et al. (2012). The detection limit however still shows that the assay is sensitive enough to detect IFN-γ caused by antigen stimulation, as was concluded by Maas *et al.* (2012).

The limit of quantification was found to be 875 pg/ml in this study. This determines the lowest possible quantity of rIFN-γ that can be picked up by the assay, with an accepted level of repeatable precision and accuracy (Shrivastava & Gupta 2011).

The diagnostic cut off for negative lions was determined to be OD_{bov} 0.03. This is a preliminary result which would require more samples to validate. While the OIE recognises the difficulties involved in validating wildlife diagnostic tests, the standards provide for lower numbers of known positive and known negative samples for diagnostic cut off determination than for other species. In assays for which validated tests for the same disease exist in the same or related species, 20 samples from known negative samples are said to be sufficient for provisional recognition of diagnostic cut-offs (OIE Terrestrial Manual 2014). This study had fifteen lions, which is 75% of the recommended number of samples.

There was sero-negativity in all lions tested from the screening tests used for FIV, FeLV and CMV. It was important to screen for these diseases in order to know the health status of the animals, and also to minimise confounding factors during testing using the LnIFN-γ assay for BTB diagnosis. Coinfection with diseases that result in immunosuppression has been suggested to interfere with assays that make use of the cell-mediated immune response (Viljoen *et al.* 2015). If, for example, FIV_{ple} infection coexists, it can result in a lower ability to pick positives for assays utilizing cell-mediated immune response (Viljoen *et al.* 2015). The confounding effect of stress has also been suggested as a possible source of interference for cell-mediated immunity based assays (de Lisle *et al.* 2002). In this study, stress of capture was minimised by manipulating the lions in their natural habitats and using general anaesthesia while samples were collected.

FeLV, FIV and CMV are in themselves important diseases that should be screened for in lion populations as they have the potential to kill animals; or cause fatal coinfections with other infections. They are also infections which can be spread by translocating animals. These populations had never been screened for these diseases although animals can be moved between properties as shown in the history of some lions. However, the CMV screening test that was used is validated for use in canine species but not in lions. This might result in false negatives from the assay.

5 CONCLUSIONS AND RECOMMENDATIONS

This study is a first step towards the validation of an ante mortem test for BTB diagnosis in lions. Further work is required to validate the assay. In order to validate the IFN- γ assay, it may be important to carry out the assay by testing more lions while using a validated BTB diagnostic test such as the intradermal skin testing concurrently, or to use the assay in known positive animals through culture. The intradermal skin test is also known to not be affected by FIV coinfection (Viljoen *et al.* 2015).

The potential of the LnIFN-γ assay as a diagnostic test for BTB is evident. It offers the potential for relatively quick testing time possibly for free-ranging lions while the animals are held as there is no difference between allowing for whole blood cells to be stimulated over 48 hours compared to the 24 hour incubation periods according to this study.

These lions were also screened for possible coinfections. No circulating antibodies to FeLV and FIV were found in the three lion populations at the time the study was performed. It cannot be concluded that CMV was not in the animal populations as the test used was not validated for use in felines; although it is commonly used by field practitioners to screen for CMV.

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133441.pdf.

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7 APPENDIX

All communications should be addressed to "Director Division of Veterinary Services"

Telephone: 263-4-706604
Fax: 263-4-791516
Email: dvts@africaonline.co.zw
Website: www.vetservices.org.zw

ZIMBABWE

Reference:

DIVISION OF VETERINARY SERVICES
DEPARTMENT OF LIVESTOCK AND VETERINARY SERVICES
Ministry of Agriculture, Mechanisation and Irrigation Development

P O Box CY 56, Causeway

Re: Approval of "Optimising the interferon gamma (IFN-γ) assay for the diagnosis of bovine tuberculosis (BTB); and screening for Feline immunodeficiency virus and canine distemper co-infections in captive lions in Zimbabwe"

Tuberculosis is an important zoonotic disease of socio- economic importance in Zimbabwe. It is also a disease of concern due to its transboundary nature and its negative effects on biodiversity.

In wild animals in Zimbabwe, it is currently diagnosed through the skin test that requires recapturing after three days; and through culture following death of animals.

This research is aimed at optimising the interferon gamma assay for the diagnosis of bTB and determining the sero-prevalence of feline immunodeficiency virus and canine distemper virus in lions. The study will be carried out in captive lion populations for the validation of the interferon gamma assay for future use as a bTB diagnostic test in wild animals.

The Division of Veterinary Services' diagnostics and research branch and the Veterinary Research committee approved Dr Kudzaishe Vhoko's research project on *"Optimising the interferon gamma (IFN-γ) assay for the diagnosis of bovine tuberculosis (BTB); and screening for Feline immunodeficiency virus and canine distemper co-infections in captive lions in Zimbabwe"* in Zimbabwe according to the attached protocol; in partial fulfilment of her Master's degree (Msc Human/animal/ecosystem health) at the University of Pretoria.

13/04/2016

DIRECTOR
Dept.of Vet. Technical Services
Livestock & Vet. Services Division
13 APR 2016
P.O. BOX CY 551, CAUSEWAY
ZIMBABWE

Dr P V Makaya

Deputy Director: Veterinary Services (Diagnostics and Research)

Chairman: Zimbabwe veterinary projects steering committee

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: 12/11/1/4/1

Dr Kudzaishe Vhoko
Department of Veterinary Services
Bevan Building
Harare
Email:kmvhoko@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 Dr Kudzaishe Vhoko

Your email dated 26 April 2016 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. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. The study must be conducted in compliance with the Veterinary and Para-Veterinary Professions Act 1982 (Act No. 19 of 1982);
4. A veterinary import permit will be required prior to the importation of lion (*Panthera leo*) serum and plasma samples;
5. A veterinary health certificate must accompany the veterinary import permit, whereby the Zimbabwean Veterinary Authority attest, that a state veterinarian was

present at the time of sample collection, to verify the species and type of samples collected and to ensure no cross contamination of the samples occurs with biological material of any other species;

6. The lion serum and plasma samples must arrive in the Republic of South Africa by air and must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and/or the National Road. Traffic Act, 1996 (Act No. 93 of 1996).

Title of research/study: Optimising the Interferon-Gamma Assay for Use in the Diagnosis of Bovine Tuberculosis (BTB) in Captive Lions (*Panthera Leo*) in Harare, Zimbabwe; and Screening for Diseases in these Lion Populations.
Researcher: Dr Kudzaishe Vhoko
Your Ref./ Project Number:
Institution: Faculty of Veterinary Science, UP
Our ref Number: 12/11/1/18

Kind regards,

MMaja

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH

Date: 2016 -07- 2 5

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

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Department Veterinary Tropical Diseases
Faculty of Veterinary Science

Consent Letter

I, Gordon Putterill of Bally Vaughan Game Farm & Sanctuary (property), give consent for blood samples to be obtained from lions on my property for the purpose of research.

I am aware that this is an MSc project by Dr Kudzaishe Vhoko. The project is titled *"Optimising the IFN-γ assay for use in diagnosis of BTB in captive lions in Harare, Zimbabwe; and screening of diseases of importance in these lion populations."*

16/9/16

SIGNED **DATE**

Para clinical Building,
University of Pretoria, Private Bag X04
ONDERSTEPOORT 0110, South Africa
Tel +27 (0)12 529 8230/8288
Fax +27 (0)12 529 8312
www.up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Optimising the interferon-gamma (IFN-γ) assay for use diagnosis of bovine tuberculosis (bTB) in captive lions *(Panthera leo)* in Harare, Zimbabwe; and screening for diseases of importance in these lion populations.**
PROJECT NUMBER	**V055-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. K Vhoko**

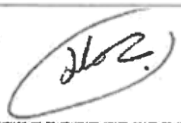
STUDENT NUMBER (where applicable)	**UP_** 15390692
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Captive lions *(Panthera leo)***	
NUMBER OF ANIMALS	**30**	
Approval period to use animals for research/testing purposes		**July 2016 – July 2017**
SUPERVISOR	**Prof. D Morar-Leather**	

Conditions: The AEC has noted that this project will be completed in a facility outside of South Africa. Since the AEC has not inspected the facility, please note that we cannot comment on the quality of the facility other than what was provided in the study questionnaire

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 27 July 2016
CHAIRMAN: UP Animal Ethics Committee	Signature 

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF AGRICULTURE
DIRECTORATE: ANIMAL HEALTH
PRIVATE BAG X 138
2016 -10- 05
PRETORIA 0001
FORESTRY & FISHERIES

Directorate of Animal Health
Import-Export Policy Unit
Private Bag X138
Pretoria, 0001
Republic of South Africa
Tel: (27)-012-319 7514
Fax: (27)-012-329 8292
PERMIT NO: 13/1/1/30/2/0-201610000699
Valid from: 2016-10-05
Expiry date: 2017-01-05

IMPORTER:
DARSHANA MORAR-LEATHER
UNIVERSITY OF PRETORIA
FACULTY OF VETERINARY SCIENCE
SOUTPAN ROAD
ONDERSTEPOORT

VETERINARY IMPORT PERMIT FOR PATHOLOGY SPECIMENS

[Issued in terms of the Animal Diseases Act, 1984 (Act No. 35 of 1984)]

Authority is hereby granted for you to import 10 LION SERUM SAMPLES / 264 LION PLASMA SAMPLES into Republic of South Africa:

From: ZIMBABWE
subject to the following conditions:

1. The consignment must be accompanied by this original permit and an original veterinary health certificate, complying with the conditions stipulated overleaf (IMP.PATH.CE.10/2013), duly completed and signed by an official veterinarian, authorised thereto by the Veterinary Authority of ZIMBABWE.
2. The specimens are to be securely packed and transported in leakproof containers, sealed by an authorised official of the Veterinary Authority of the exporting country;
3. The specimens must be kept and used for purposes of testing/research at the laboratories of UNIVERSITY OF PRETORIA, FACULTY OF VETERINARY SCIENCE, DEPARTMENT OF VETERINARY TROPICAL DISEASES under the personal supervision of DR DARSHANA MORAR-LEATHER;
4. On completion of tests/research the specimens, including all contaminated/infectious things or animal products (as defined by the Animal Diseases Act, 1984 [Act No. 35 of 1984]) derived/produced from or that came into contact with the above-mentioned specimens, must be destroyed by incineration. Records of the incinerations must be maintained for a period of 5 years, and made available for auditing to the Veterinary Authority upon request.
5. The consignment must be airfreighted through port of entry OR TAMBO INTERNATIONAL AIRPORT. **Samples may only be imported as manifest cargo under an airwaybill number and may not be imported as personal luggage.**
6. The consignment must be accompanied by this permit and its arrival reported immediately to the inspecting veterinary official: KEMPTON PARK Tel: 011 973 2827, and may not be released without his/her written permission.
7. Upon arrival the inspecting veterinary official will inspect the consignment and release it to the importer only after he/she is satisfied that all the import conditions have been complied with in full.
8. **This permit does not absolve the importer from compliance with the provisions of any other legislation relating to this import.**
9. This permit is subject to amendment or cancellation by the Director Animal Health at any time and without prior notice being given.
10. This permit is valid for three (3) months from date of issue and FOR ONE CONSIGNMENT ONLY.

SPECIAL CONDITIONS:
IN ADDITION, THE VETERINARY HEALTH CERTIFICATE (DESCRIBED IN CONDITION 1 ABOVE) ISSUED BY A VETERINARIAN AUTHORISED THERETO BY THE VETERINARY AUTHORITIES OF ZIMBABWE MUST STATE THAT THE SAMPLES ARE FROM LION ONLY AND HAVE NOT BEEN CONTAMINATED WITH ANIMAL PRODUCT OF ANY OTHER SOURCE

pp abraham
DIRECTOR: ANIMAL HEALTH

NOTE:
- All imports for research purposes require Section 20 permission in compliance with the Animal Diseases Act.

(IMP.PATH.CE.10/2013)

- Any consignment imported into South Africa packed with either wood packaging material or dunnage, will require treatment to remove any pests present (by heat or methyl bromide fumigation). Treatment must be indicated as per IPPC prescript on wood packaging material. [Directorate: Inspection Services Tel: 012 309 8754 or Fax 086 732 4768 or www.daff.gov.za]

VETERINARY HEALTH CERTIFICATE FOR PATHOLOGY SPECIMENS FOR LABORATORY USE FOR EXPORT TO THE REPUBLIC OF SOUTH AFRICA

CERTIFICATE NUMBER [1]: VB/2/13/142

RESPONSIBLE VETERINARY AUTHORITY: DR E.O. NANIWA.

ISSUING VETERINARY AUTHORITY: DPT OF LIVESTOCK & VETERINARY SERVICES - ZIMBABWE

SOUTH AFRICAN VETERINARY IMPORT NUMBER: 13/1/1/80/2/0-20161000699

A. IDENTIFICATION OF CONSIGNMENT

1. **Description of the products:**
   10 LION SERUM SAMPLES
   264 LION PLASMA SAMPLES

2. **Type and number of packages:**
   5 BOXES CONTAINING PLASMA & SERUM TUBES

3. **Net weight of consignment:**

4. **Identification marks on the packages (eg: lot/batch numbers):**

B. ORIGIN OF CONSIGNMENT:

1. **Name and Address of facility where product is manufactured:**
   CENTRAL VETERINARY LABORATORY
   18A BEVAN BUILDING, BORROWDALE RD
   P O BOX CY551 CAUSEWAY, HARARE

2. **Name and Address of exporter:**

DR K VHOKO
Central veterinary Laboratory, P.O Box 551 Causeway
Harare

CERTIFICATE NUMBER [1]: VB/2/13/142

C. DESTINATION OF CONSIGNMENT:

1. **Name and address of Consignee:**

Dr. Darshana Morar-Leather (Room 2-20.1)
Paraclinical Building, Dept. Vet. Tropical Diseases
Faculty of Vet. Science, University of Pretoria,
ONDERSTEPOORT - 0110 - SOUTH AFRICA

2. **Means of transport (flight no/vessel name):**

Air transport

D. HEALTH ATTESTATION

I, the undersigned official veterinarian, hereby certify that the products described in part A of this document were securely packed in leakproof containers, sealed by an authorised official of the Veterinary Authority of the exporting country and bear the following seal numbers:

Issued at: DLVS on 07/10/16

Signature of Official Veterinarian [2]

Official stamp [2]

DIRECTOR
DEPARTMENT OF VETERINARY
TECHNICAL SERVICES
LIVESTOCK & VET. SERVICES DIVISION
- 7 OCT 2016
P.O. BOX CY 551, CAUSEWAY
HARARE ZIMBABWE

Name in print: E O. WANIWA

Designation: A/Import & Export officer

Address: 18 BORROWDALE RD
HARARE

(IMP.PATH.CE.10/2013)

Anigen Rapid CDV Ag Test Kit

Vet Diagnostic

■ Principles

The Anigen Rapid CDV Ag Test Kit is a chromatographic immunoassay for the qualitative detection of Canine Distemper virus antigen in conjunctiva, urine, serum or plasma.

The Anigen Rapid Canine Distemper virus Ag Test Kit has a letter of "T" and "C" as test line and control line on the surface of the device. Both the test line and control line in result window are not visible before applying any samples. The control line is used for procedural control. Control line should be always appeared. If the test procedure is performed properly and the test reagents of control line are working. A purple test line will be visible in the result window if there is enough Canine Distemper virus antigen in the specimen.

The specially selected Canine Distemper virus antibodies are used in test band as both capture and detector materials. These enable the Anigen Rapid CDV Ag Test Kit to identify Canine Distemper virus antigen in conjunctiva, urine, serum or plasma with a high degree of accuracy.

■ Materials provided (10 tests/kit)

1) Ten(10) Anigen Rapid CDV Ag Test Kits
2) Ten(10) Specimen tubes containing assay diluent buffer
3) Ten(10) Sample collection swabs
4) Ten(10) Disposable droppers
5) One(1) Instruction for use

■ Precautions

1) For veterinary diagnostic use only.
2) For best results, strict adherence to there instructions is required.
3) All specimens should be handled as being potentially infectious.
4) Do not open or remove test kit from their individually sealed pouches until immedia- tely before their use.
5) Do not use the test kit if the pouch is damaged or the seal is broken.
6) Do not reuse test kit.
7) All reagents must be at room temperature before running the assay.
8) Do not use reagents beyond the stated expiration date marked on the label.
9) The components in this kit have been quality control tested as standard batch unit.
Do not mix components from different lot numbers.

■ Storage and Stability

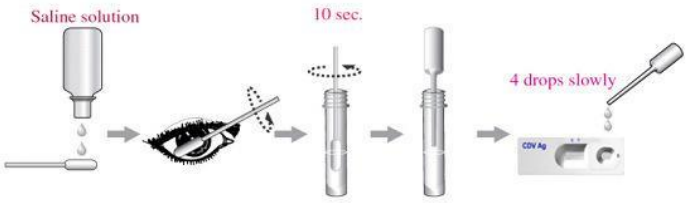
The kit can be stored at room temperature(2~30℃) or refrigerated. The test kit is stable through the expiration date marked on the package label. **DO NOT FREEZE**. Do not store the test kit in direct sunlight.

■ Specimen Collection and Preparation

1) The test should be performed using the canine secretion of eye(the conjunctiva), saliva, urine, serum or plasma.
2) After collecting the specimen using swab, the specimen should be immediately extracted and tested.
3) If specimens are not immediately tested, they should be refrigerated at 2~8℃, For storage not less than 48 hours, freeze the specimen at -20℃ or below.

■ Procedure of the test

1) Collect the samples from conjunctiva or urine using the sample collection swab pre-wetted with saline solution. In case of serum or plasma samples, you can use the dropper.
2) Insert the swab into the specimen tube containing 300ul of assay diluent.
In case of serum or plasma samples, add 2-3 drops of the serum or plasma into the specimen tube containing 300ul of assay diluent using the dropper.
3) Mix the swab samples with assay diluent to extract well.
4) Remove the test device from the foil pouch, and place it on a flat and dry surface.
5) Add four (4) drops of the mixed sample into the sample hole using the dropper, drop by drop and slowly
6) As the test begins to work, you will see purple color move across the result window in the center of the test device. If the migration has not appeared after 1 minute, add one more drop of the mixed sample to the sample well.
7) Interpret test results at 5-10 minutes.



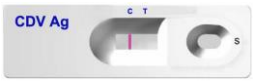
[Figure for test procedures]

■ Interpretation of the test

A color band will appear in the left section of the result window to show that the test is working properly. This band is the control band. The right section of the result window indicates the test results. If another color band appears in the right section of the result window. This band is the test band.

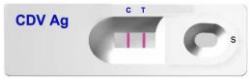
1) Negative result

The presence of only one band within the result window indicates a negative result.



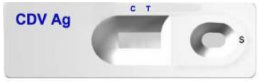
2) Positive result

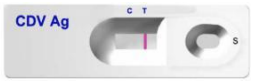
The presence of two color bands ("T" and "C") within the result window, no matter which band appears first indicates a positive result.



3) Invalid Result

If the purple color band is not visible within the result window after performing the test, the result is considered invalid. The directions may not have been followed correctly or the test may have deteriorated. It is recommended that the specimen be re-tested.





■ Limitations of the test

Although the Anigen Rapid Canine Distemper virus Ag Test kit is very accurate in detecting Canine Distemper virus antigen, a low incidence of false results can occur. Other clinically available tests are required if questionable results are obtained. As with all diagnostic tests, a definitive clinical diagnosis should not be based on the results of a single test, but should only be made by the veterinarian after all clinical and laboratory findings have been evaluated.

■ Bibliography of suggested reading

1) Tsuyoshi GEMMA, Naoko MIYASHITA, Yeon-Sil SHIN, Masatsuge OKITA, Takeshi MORI, Kiyoco IWATSIKI, Takeshi MIKAMI and Chieko KAI " Serological Survey of Cnine Distemper Virus Infection Using enzyme Linked Immunosorvent Assay " J. Vet. Med. Sci.57(4) : 761~63, 1995

2) Yeon-Sil SHIN, Takeshi MORI, Masatsuge OKITA, Twuyochi GEMMA, Chieko KAI and Takeshi MIKAMI "Detection of Canine Distemper Virus Nucleocapsid Protein Gene in Canine Peripheral Blood Mononuclear Cells by RT-PCR" J Vet. Med Sci. 57(3) : 439-445, 1995

3) Jeremiah T.Saliki and Terry W. Lehenbauer " Monoclonal Antibody- Based Competitige Enzyme- Linked Immunosorbent Assay for Detection of morbillibirus Antibody in Marine Mammal Sera " Journal of Clinical Microbiology, Vol. 39, No. 5, May 2001, P. 1877~1881

4) Veronika von Messling, Timm C. Harber, Volker Moenning, Peter Rautenberg, Ingo Molte and Ludwig Haas "Rapid and Sensitive Detection of Immunglobulin M(IgM) and IgG antigodies againse Canine Distemper Virus by a New Recombineant Nuclecapsid Protein – Based Enzyme-Linked Immunosorbent Assay" Journal of Clinical Microbiology Vol. 37, No. 4 Apr. 1999, P. 1049~1056

5) Tsuyochi GEMMA, Kiyoko IWATSIKI, Ueon-Sil SHIN, Emi YOSHIDA, Chieko KAI, and Takechi MIKAMI "Serological Analysis of Canine distemper Viurs Using an Immunocapture ELISA" J. Vet. Med. Sci.58 (8) : 791~794, 1996

Doc. No. : I 1103-3

Issued date : Jan. 30, 2009

BIONOTE

Vet Diagnostic

Anigen Rapid FIV Ab/FeLV Ag Test Kit

Vet Diagnostic

■ Principles

The Anigen Rapid FIV Ab/FeLV Ag Test Kit is a chromatographic immunoassay for the qualitative detection of Feline Leukemia virus antigen and Feline Immunodeficiency virus antibody in feline serum, plasma or whole blood.

The Anigen Rapid Feline Leukemia virus Ag/Feline Immunodeficiency virus Ab Test Kit has the letters "T" and "C" as the Test line and Control line on the surface of the device. Both the test line and control line in the result window are not visible before applying any samples. The control line is used for procedural control, and should always appear if the test procedure is performed properly and the test reagents of the control line are working. A purple test line will be visible in the result window if there is enough Feline Leukemia virus antigen and/or Feline Immunodeficiency virus antibody in the specimen.

The specially selected Feline Immunodeficiency virus antigen and Feline Leukemia virus antibody are used in the test band as both capture and detector materials. These enable the Anigen Rapid FIV Ab/FeLV Ag Test Kit to identify Feline Leukemia virus antigen and Feline Immunodeficiency virus antibody in feline serum, plasma or whole blood with a high degree of accuracy.

■ Materials provided (10tests/kit)

1) Ten(10) Anigen Rapid FIV Ab/ FeLV Ag Tests.
2) One(1) Bottle containing 6 ml of assay diluents.
3) Ten(10) Disposable Capillary tube for specimens.
4) Ten(10) Anticoagulant bottles.
5) One(1) Instructions for use.

♣ A dark color score line on the capillary tube is the indicator line for 10㎕.

■ Precautions

1) For veterinary diagnostic use only.
2) For best results, strict adherence to the instructions is required.
3) All specimens should be handled as being potentially infectious.
4) Do not open or remove the test kits from their individually sealed pouches until immediately before their use.
5) Do not use the test kit if the pouch is damaged or the seal is broken.
6) Do not reuse test kits.
7) All reagents must be at room temperature before running the assay.
8) Do not use reagents beyond the stated expiration date marked on the label.
9) The components in this kit have been quality control tested as a standard batch unit. Do not mix components from different lot numbers.

■. Storage and Stability

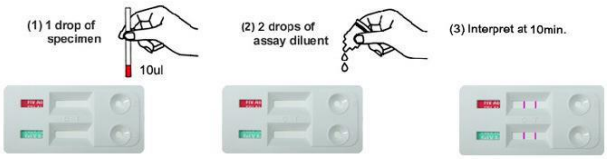
The kit can be stored at room temperature (2~30) or refrigerated. The test kit is stable through the expiration date marked on the package label. **DO NOT FREEZE**. Do not store the test kit in direct sunlight.

Collect and prepare serum samples using standard clinical l raboratory procedures. Serum samples may be stored refrigerated (2~7℃) for up to 72 hours; for longer storage, freeze at or below -20℃ in vials with air-tight seals.

■ Procedure of the test

1) Remove the test device from the foil pouch, and place it on a flat and dry surface.
2) Using the disposable capillary tube , add one (1) drop (approximately 10ul) of feline serum, plasma or whole blood into the sample hole, and then add two (2) drops (approximately 60ul) of the assay diluents.
3) As the test begins to work, you will see a purple color move across the result window in the center of the test device. If the migration has not appeared after 1 minute, add one more drop of the assay diluents to the sample well.
4) Interpret test results at 10 minutes. Do not interpret after 10 minutes.

[Figure for test procedures]



■ Interpretation of the test

A color band will appear in the left section of the result window to show that the test is working properly, this band is the control band. The right section of the result window indicates the test results. If another color band appears in the right section of the result window, this band is the test band.

1) Negative result

The presence of only one band within the result window on both of the FIV Ab and FeLV Ag test areas indicates a negative result.

2) Simultaneous FIV Ab and FeLV Ag Positive result

The presence of two color bands ("T" and "C") within the result window on both of the FeLV Ag and FIV Ab test areas respectively, no matter which band appears first, indicates a positive result of Feline Leukemia virus Ag and Feline Immunodeficiency virus Ab simultaneously.

3) FIV Ab Positive result

The presence of two color bands ("T" and "C") within the result window on the FIV Ab test area, and the presence of only one band ("C") within the result window on the FeLV Ag test area, no matter which band appears first, indicates a positive result of Feline Immunodeficiency virus Ab.

■. Specimen Collection and Storage

1) The test should be performed using serum, plasma, or whole blood.

2) [Whole blood]

Collect on anticoagulated blood sample in EDTA, heparin or citrate using standard clinical laboratory procedures. Anticoagulated whole blood samples should be tested within 24 hours of drawing. If delays are expected, samples should be stored either on ice or refrigerated(2~7℃), but should not be frozen. If anticoagulated whole blood samples cannot be tested within this period of time, separate plasma by centrifugation and store as described in the next section.

3) [Plasma]

Collect an anticoagulated blood sample using standard clinical laboratory procedures. Separate plasma by centrifugation. Plasma samples may be stored refrigerated(2~7℃) for up to 72hours; for longer storage, freeze at or below -20℃ in vials with air-tight seals.

4) [Serum]

4) FeLV Ag Positive result

The presence of two color bands (“T” and “C”) within the result window on the FeLV Ag test area, and the presence of only one band (“C”) within the result window on the FIV Ab test area , no matter which band appears first, indicates a positive result of Feline Leukemia virus Ag.

5) Invalid result

If the purple color band is not visible within the result window after performing the test, the result is considered invalid. The directions may not have been followed correctly or the test may have deteriorated. It is recommended that the specimen be re-tested.

■ Limitations of the test

Although the Anigen Rapid FIV Ab/FeLV Ag Test Kit test kit is very accurate in detecting Feline Immunodeficiency virus antibody and/or Feline Leukemia virus antigen, a low incidence of false results can occur. Other clinically available tests are required if questionable results are obtained. As with all diagnostic tests, a definitive clinical diagnosis should not be based on the results of a single test, but should only be made by the veterinarian after all clinical and laboratory findings have been evaluated.

Doc. No. : 1105-1
Issued date : Jane. 8, 2008

BIONOTE

Vet Diagnostic

Lion Physical Examination

FIELD NO: .....

A) TB Lesions

Ulcerated Skin Lesions | Yes | No |

Positions .....

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Samples — **No. collected**

Biopsies | Yes | No | .....

Swab | Yes | No | .....

Acid Fast smears | Yes | No | .....

Hygromas

Right | Yes | No |

Left | Yes | No |

Comments

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Enlarged Lymphnodes

Mandibular | Yes | No |

Prescapular | Yes | No |

Auxillary | Yes | No |

Inguinal | Yes | No |

Popliteal | Yes | No |

Other | Yes | No |

Comments

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Periostitis/Osteomyilitis | Yes | No |

Bones Affected:

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Comments

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Wounds | Yes | No |

Bites No: .....

Scratches No: .....

Description

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Other | Yes | No |

Comments

.....

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B) FIV

Skin coat | Shiny, smooth | Dull, listless | Mange |

Dehydration | Normal | 30% | Other..... |

Gingivitis score | 1 | 2 | 3 | 4 |

Papillomas | Present | Absent |

Tick count Periocular [ ] Perianal [ ]

Photo taken | Yes | No |

Ocular Lesions: .....

Pregnancy .....

Lactation | Yes | No |

Other Comments:

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