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FELINE LENTIVIRUS: MOLECULAR ANALYSIS AND EPIDEMIOLOGY IN SOUTHERN AFRICAN LIONS (*Panthera leo*)

A Dissertation

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Abstract:

Feline immunodeficiency virus is a retrovirus of domestic cats causing significant lifelong infection. Infection has also been detected in nondomestic species, including African lions. It is endemic in certain populations in east and southern Africa. Infection leads to immunologic dysfunction and immunosuppressive disease in domestic cats; however, little research exists about the pathogenic effects of infection in lions and its epidemiological impact on free-ranging and captive populations. Little is known about the lentivirus in these populations at the molecular and host level. Analysis of the virus from these populations is necessary for development of detection assays that are both sensitive and specific.

Whole-blood and serum samples were collected opportunistically from free ranging lions in Kruger National Park, South Africa, and from Hlane Royal National Park, Swaziland. Whole-blood and serum samples were also collected from captive exotic felids in RSA andUS and domestic cats in RSA. A nested polymerase chain reaction assay was performed on all whole-blood samples, and all positive products were sequenced and analyzed phylogenetically. Serum samples were tested for cross-reactive antibodies to domestic feline lentivirus antigens and cross-reactive antibodies to puma lentivirus synthetic envelope peptide antigen. Serum samples were tested for feline haptoglobins and feline alpha-one acid glycoproteins by radial immunodiffusion.

This research represents the first epidemiological study of the lion lentivirus among free-ranging lions of Kruger National Park and the first epidemiological study comparing genetic material to antibody-based methods of lentivirus detection on lions in RSA. The polymerase chain reaction assay was successful in amplifying

the lion lentivirus from African lions.

The conservation management of free-ranging lions must consider the infectious agents to which they are susceptible. No conclusions can be drawn from this investigation with respect to the potential virulence or pathogenic distinctions between viral subtypes, as little is known about the definitive consequences of lion lentivirus infection in African lions. Immunologic studies may uncover potential differences in immune expression among lentivirus-positive and -negative individuals with regard to increased resistance or increased susceptibility to infection or pathogenicity. To this end, lion lentivirus infection status could also be compared to the infection status of bovine tuberculosis.

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List of Abbreviations

FIV: Feline immunodeficiency virus
AIDS: Acquired immune deficiency syndrome
FIVfca: Domestic cat lentivirus
FIVple: African lion lentivirus
FIVpco: Puma lentivirus
Gag: Group specific antigen
Pol: Polymerase
Env: Envelope
PBMC: Peripheral blood mononuclear cells
PCR: Polymerase chain reaction
RT-PCR: Real-time polymerase chain reaction
ELISA: Enzyme-linked immunosorbent assay
KNP: Kruger National Park
RSA: Republic of South Africa
APP: Acute phae proteins
Hp: Haptoglobin
AGP: Alpha-1 acid glycoprotein
Tb: Tuberculosis
KNP-EPI-N: Kruger National Park Epidemiology subset, north section
KNP-EPI-S: Kruger National Park Epidemiology subset, south section
LP: The Lion Park
Jhb: Johannesburg Zoo
Pta: Pretoria Zoo
DC: Domestic cat
StL: St. Louis Zoo
BCS: Body condition score
GS: Gold standard
CTP: Combined test prevalence
OR: Odds ratio

Part One

LITERATURE REVIEW

1. LITERATURE REVIEW

1.1 Introduction to the Viruses of the Genus *Lentivirus,* Including the African Lion Lentivirus

Lentiviruses are members of the family *Retroviridae* that have been known for many years to infect a variety of domestic animals, including domestic cats. The feline immunodeficiency virus (FIV) of domestic felids (*Felis catus*) was first isolated by Pedersen *et al.* in 1986 from a domestic cat with a history of chronic opportunistic infections that resembled the acquired immune deficiency syndrome (AIDS) in humans (Pedersen, 1987). There are several distinct feline lentiviruses in non-domestic felids that are related to the domestic feline lentivirus (Burkhard 2003). Serological evidence of a feline lentivirus among free-ranging lions was first reported in the early 1990s (Lutz 1992; Olmsted, 1992; Spencer, 1992). A lion lentivirus was first isolated and documented by Brown *et al.* in 1994, along with partial sequences of several cloned isolates. Antibodies reactive to domestic cat lentiviruses were found as early as 1968 in banked serum samples of domestic cats in the United States and Japan (Pedersen 1987; Shelton 1989; Furuya 1990; Bendinelli 1995; Kanzaki 2004). Antibodies reactive to lion lentiviruses appeared as early as 1977 in lions in the Kruger National Park in South Africa, and in the Etosha National Park in Namibia (Spencer 1992).

The African lion lentivirus is a virus of the genus *Lentivirus*, in the subfamily *Lentivirinae*, and is a member of the family *Retroviridae* (Pedersen 1987; Miyazawa 1994). It is related to both the domestic and other non-domestic feline lentiviruses. The feline lentiviruses are more closely related phylogenetically to the ungulate lentiviruses of domestic animals such as the equine infectious anemia virus (EIAV), maedi-visna

virus of sheep, and caprine arthritis encephalitis virus (CAEV), than to the primate lentiviruses such as simian immunodeficiency virus (SIV) and the human immunodeficiency virus (HIV), when comparing amino acid sequences of the reverse transcriptase (RT) gene (Goff 2001). It has been suggested that this relationship is demonstrated in the ability of both feline and ungulate lentiviruses to infect both dividing as well as non-dividing cells, whereas the primate lentiviruses only infect dividing cells (Poeschla 1998).

The nomenclature for this group of viruses has evolved as more information has been published about the individual isolates. The term FIV is typically used when referring to feline lentiviruses of domestic cat origin. However, as lentivirus strains originating from other non-domestic feline species were later identified, it became necessary to develop nomenclature more specific to each strain. Thus an abbreviation of the genus and species of the feline host is now used primarily in the scientific literature as a subscript to FIV. For example, domestic cat (*Felis catus*) FIV becomes FIVfca, African lion (*Panthera leo*) FIV is designated FIVple, puma (*Puma concolor*) FIV isolates are referred to as FIVpco (pumas are also referred to as cougars, mountain lions, or panthers), and so on (VandeWoude 2006). In addition to this terminology, the lion and puma lentiviruses are also referred to as LLV and PLV, respectively, a terminology more commonly used prior to the above described nomenclature (Olmsted, 1992, VandeWoude, 1997).

Much attention has been paid to FIVfca due to its clinical and pathologic similarities to HIV. As a result, FIVfca is often used as a small animal model of HIV infection and AIDS in humans. In contrast to the wealth of information on HIV and

FIVfca, however, little is known about the lion lentiviruses, particularly in free-roaming populations of lions. There are many publications on non-domestic feline lentivirus studies, particularly reports of seroprevalence among free-roaming populations, yet only a handful of publications exist that are specific to lion lentiviruses. As a result, a certain amount of extrapolation from the FIVfca literature is necessary in order to begin any comprehensive study of the lion lentiviruses, as is the case in this literature review.

1.2 Virus Characteristics

Retroviruses are a diverse group of viruses found in all vertebrates. Retroviruses are unique in that the infectious viral RNA is reverse transcribed into a proviral dsDNA form that is integrated into the host cellular DNA. The integrated proviral DNA serves as the template for the formation of viral RNAs, and the proteins responsible for virus assembly (Goff 2001). The presence of an integrated DNA stage of the virus enables it to more effectively evade host immune defenses, thus maintaining a persistent, lifelong infection in an individual host (Pedersen 1987; Yamamoto 1989). The lentiviruses are unstable RNA viruses with high rates of mutation due to the lack of proofreading enzyme (Pedersen 1987; Goff 2001). This results in a high error rate by the viral polymerase during DNA synthesis, and leads to the rapid evolution of genetic variants during replication in the host (Doolittle 1989; Gojobori 1990; Johnson 1991; Salminen 1993).

The mature virion measures 105-125 nm in diameter (Pedersen 1987). The virions have a spherical to ellipsoid morphology, and are surrounded by an envelope consisting of a lipid membrane bilayer with glycoprotein projections, giving it a spiked appearance when viewed by electron microscopy (Pedersen 1987; Goff 2001). The

nucleocapsid core is cylindrical or conical (Goff 2001). The viral genomic RNA is present as a highly condensed homodimer of two identical nucleotide sequences, thus it is functionally diploid. The dimer is linked together by an interaction between the two 5' ends of the RNA strands in a self-complementary region known as the dimer linkage structure. The viral genome is a linear, positive-sense RNA that is approximately 9,200 nucleotides long (Goff 2001).

Lentivirus genomes contain three major genes, along with a variable amount of small regulatory or accessory protein genes. The major genes, or open reading frames (ORFs) from 5' to 3' are the group-specific antigen (Gag), polymerase (Pol), and envelope (Env) genes, flanked on either side by long terminal repeat (LTR) sequences (Pedersen 1987; Goff 2001). Each LTR contains an untranslated 3' end with a polyadenylated string sequence of approximately 200 base pairs, a capped 5' end, and a series of repeat sequences in between. These regions function in various regulatory roles such as primer binding, initiation of minus-strand DNA synthesis, and viral RNA packaging (Goff 2001). The Gag gene encodes the matrix (MA), capsid (CA), and nucleocapsid (NC) proteins. The Pol gene encodes the protease (PR), reverse transcriptase (RT), and integrase (IN) proteins. The Env gene encodes the surface (SU) and transmembrane (TM) envelope glycoproteins. The Env gene product is responsible for the virus' receptor-mediated tropism, fusion activity, and for eliciting a host cell immune response (Pedersen 1987; Goff 2001). Consequently, the Env region has the most nucleotide variability of the entire genome, which can be as high as 20% among isolates of FIVfca from domestic cats (Bendinelli 1995, Kanzaki 2004).

Virus Receptors and Cell Entry.--Retroviruses gain entry into a cell through a series of interactions with the cell's surface receptors. They employ a variety of host cell surface receptors in order to gain entry into a cell, and this variety of receptors may be partially responsible for the differences in viral pathogenicity observed among the felines susceptible to lentivirus infection (Smirnova 2005). Among the lentiviruses, HIV in humans is the most studied. The tropism of HIV-1 infection involves CD4, a surface protein found on T cells of the immunologic system, and more specifically found on helper T cells and macrophages. CD4 was once theorized as the primary lentivirus cell receptor that mediates virus binding to the cell surface, as the cellular tropism for FIVfca was similar to that of HIV in humans; however several studies have reported that it is not used as a primary or co-receptor for FIVfca entry into the host cell (Hosie 1993; Norimine 1993; Willett 1999).

Although it is not entirely clear what receptors are utilized by feline lentiviruses for host cell entry, CD4 does not appear to be a primary nor a co-receptor (Brown 1991; Hosie 1993; Norimine 1993; Pedersen 1993; Bendinelli 1995; Willett 1999). Research investigating the possibility of the chemokine receptor CXCR4 as a possible receptor or co-receptor for envelope- mediated FIVfca fusion found this to be the case for primary isolates of FIVfca clade A strains, and laboratory adapted isolates of clade B strains (Egberink 1992; Willett 1997b; Poeschla 1998; Richardson 1999; Frey 2001). It is now confirmed that this chemokine receptor mediates virus entry for all clades of FIVfca, and some FIVfca primary and cell culture adapted strains can infect cells using CXCR4 as the sole receptor (Lerner 2000; VandeWoude 2006). CXCR4 expression is found among activated T cells, B cells, and monocytes in domestic cats (Willett 2003). Thus the host

cellular tropism for primary FIVfca isolates are mitogen-activated peripheral blood mononuclear cells (PBMCs), dendritic cells, macrophages, thymocytes, and IL-2 dependent T cell lines (Brown 1991; English 1993; Dean 1996; Dow 1999; Vahlenkamp 1999).

The tumor necrosis factor receptor CD134, a helper T cell activation antigen, is also a binding receptor for FIVfca that induces a conformational change in the V3 (variable) region of the viral Env gene product that allows entry via CXCR4, a mechanism similar to that described for HIV (Vahlenkamp 1997; Willett 1997a; Willett 1997b; de Parvesal 2004; Shimojima 2004; de Parvesal 2005; Willett 2006). CD134 expression is primarily restricted to CD4+ T lymphocytes; however, in humans and mice it has also been found to be expressed at lower levels on activated CD8+ T cells, macrophages, and activated B cells (Paterson 1987; Baum 1994; Durkop 1995; Al-Shamkhani 1996; Stüber 1996; Gramaglia 1998).

The interactions between viral Env proteins and cell receptors are complex, and involve an initial binding of virus to the host cell membrane by the SU subunit of the Env glycoprotein. This induces a conformational change in the Env protein, followed by fusion of the viral and host cellular membranes initiated by the TM glycoprotein encoded by the Env gene, and an internalization of the viral core into the host cellular cytoplasm (Goff 2001). The cellular tropism of FIVfca is partly determined by the variability of amino acids in the Env glycoproteins (Verschoor 1995; Vahlenkamp 1997).

The receptors for non-domestic feline lentiviruses have not been definitively characterized, and appear to differ from those identified in FIVfca infection (Smirnova 2005). An experimental infection of a domestic cat with a FIVpco isolate reported a

tropism for the gastrointestinal mucosa and associated lymphoid tissue, as these tissues had the highest proviral burdens detected using a quantitative real-time polymerase chain reaction assay (RT-PCR). This study highlights the difference in tissue tropism for non-domestic feline lentivirus infections as compared to FIVfca, as some of them may be primarily enterotropic rather than lymphotropic (Terwee 2005). Similar differences have been documented in the HIV literature, as it has been reported that the simian immunodeficiency virus (SIV) provirus load is highest in the gastrointestinal tract of the primate host, with SIV and HIV holding a similar genetic ancestry to that of nondomestic feline lentiviruses and FIVfcas (Li 2005).

Reverse Transcription.--Reverse transcription of the viral RNA genome into double-stranded DNA begins once the virus has entered into the host cell cytoplasm. Initiation of the process begins at the primer binding site, where a primer tRNA anneals to the complementary viral RNA and synthesizes DNA from the primer, using the plus-strand RNA genome as template to form minus-strand DNA sequences (Goff 2001). DNA synthesis proceeds towards the 5' end of the viral RNA to generate a short complementary sequence known as the minus-strand strong-stop DNA, thus a RNA:DNA hybrid is formed. Once the hybrid is formed, RNA becomes susceptible to degradation by the RNase H activity of RT, allowing the strong-stop DNA sequence to translocate, or jump from the 5' to anneal to the 3' end of the genome (Tanese 1991; Blain 1995). There is evidence to show that this translocation occurs in *trans*, from one RNA template to the other RNA in the virion, however, evidence also supports that this translocation occurs at random, to either RNA (Yu, 1998). Once annealed, RT elongates the minus-strand strong-stop DNA to form a long minus-strand sequence. The hybrid RNA is

degraded by RNase H except for a short, purine-rich sequence near the 3' end of the genome that is RNase resistant at this time. This sequence, a short polypurine tract (ppt), remains hybridized to the minus-strand DNA, thus serving as the primer for synthesis of the plus-strand strong-stop DNA, using the minus-strand DNA as a template (Ilyinskii 1998; Robson 1999). Once the plus-strand DNA is elongated, the tRNA primer is removed at its 3' end, allowing the plus-strand DNA to pair with the 3' end of the minus-strand DNA. These sequences anneal and form a circular intermediate, with both 3' ends ready for elongation. As elongation progresses within the circle, a linear DNA is formed (Goff 2001).

Nuclear Entry and Integration of the Provirus.--Lentiviruses have the ability to infect nondividing cells, requiring active transport of the viral DNA through the intact nuclear membrane (Weinberg 1991; Bukrinsky 1992; Lewis 1992; Saib 1997). Insertion into the host cellular DNA is mediated by the viral integrase (IN) protein (Panganiban 1984; Schwartzberg 1984; Quinn 1988). This integration of the linear DNA, or provirus, into the host cellular DNA, is permanent, and results in a persistent lifelong infection of the virus in the host cell (Pedersen 1993). Upon provirus integration, host enzymes assist in virus transcription and translation, beginning with the synthesis of viral RNAs and proteins, and the assembly of progeny virions (Goff 2001).

Viral RNA Synthesis.--Viral RNA synthesis from the proviral DNA results in a long primary transcript that is processed and may be spliced to form a number of stable transcripts. These transcript RNAs are then exported to the nucleus for translation (Goff 2001).

Translation and Protein Processing.--The feline lentivirus genome contains open reading frames designated as the Gag, Pol, and Env genes. These genes are expressed to form precursor proteins which are processed by proteolytic cleavage during and after virion assembly to form mature virus proteins (Goff 2001).

Virion Assembly.--Virion assembly and RNA packaging are mediated primarily by the Gag precursor protein, and specifically by the nucleocapsid portion of Gag (Oertle 1990). The Gag precursor is cleaved by the viral protease (PR), which is expressed in Pol proteins. Gag is then processed to form the: membrane-associated, or matrix protein (MA), which binds to the inner face of the membrane and may make contact with the Env protein; the capsid protein (CA) which forms the shell of the condensed inner core of the mature virus; and the nucleocapsid protein (NC) which coats the viral RNA and participates in virion assembly (Remy 1998). The Pol precursor is processed to give rise to the protease (PR), reverse transcriptase (RT), and integrase (IN) proteins, whose functions have been previously outlined. Finally, cleavage of the Env protein gives rise to the surface (SU) and transmembrane (TM) subunits, both essential for virus infectivity (Perez 1987; McCune 1988). The SU subunit is heavily glycosylated, which may serve to enhance virus infectivity by masking surface peptides present on the virus envelope from neutralizing antibodies present in the host. SU is the major receptor binding site and is thus a determinant of the virus' host range. The TM subunit links to the SU subunit through noncovalent bonds, and is believed to play a role in the fusion of the virus with the host membrane (Freed 1990; Helseth 1990; Denesvre 1995; Hernandez 1998; Salzwedel 1999).

Virus maturation takes place at the plasma membrane, resulting in round virus particles that, upon release from the cell, change to a more condensed cylindrical or conical, enveloped structure (Goff 2001).

Feline lentivirus infections are believed to ultimately result in host cell death, however the mechanisms by which infection leads to cell death are not yet clear, although evidence supports a strong similarity in the pathogenesis of FIV and HIV (Goff 2001; Paillot 2005). One study suggests that upregulation of gene expression may facilitate host cell death in HIV (Somasundaran 1988).

1.3 Virus Diversity and Heterogeneity

Genetic diversity in feline lentiviruses can arise through one, or a combination of four phenomena: 1) by virus recombination following replication in the host; 2) by the evolution of a virus quasispecies within an infected host; 3) by virus recombination following dual infection in a host, also known as co-infection; or 4) by superinfection (Okada 1994; Sodora 1995; Kyaw-Tanner 1996).

Recombination.--Because retrovirus virions consist of two copies of RNA, either strand, or a combination of both strands known as recombination, can be used during reverse transcription to synthesize DNA (Hu 1990; Stuhlmann 1992). Recombination is possible during minus or plus-strand DNA synthesis, and occurs at a frequency as high as once per replication event per genome, on average (Zhang 1993). There are two possible ways for this to occur. The first way is known as the copy choice model, where recombination occurs during minus-strand synthesis. The second way is known as the strand-displacement assimilation model, where recombination occurs during plus-strand

DNA synthesis. In the copy choice model, a template switch occurs in which an incomplete DNA copied from one template serves as a primer to further elongation on the other RNA strand (Luo 1990; Peliska 1992). In the strand displacement model, multiple plus-strand fragments are elongating on the minus-strand template, and if a strand is displaced, the 5' end of the fragment is exposed. If a second minus-strand DNA has been synthesized, it can pair with the other minus-strand DNA to form a new DNA hybrid (Junghans 1982).

Quasispecies.--The rapid evolution of lentiviruses through nucleotide substitutions, insertions, and deletions in an individual host gives rise to a viral quasispecies, which is well documented in human HIV infection (Cichutek 1992; Pedroza-Martins 1992; Eigen 1990; Brown 1994). This phenomenon results in multiple viral variants in an individual host that continue to gather mutations in vivo, resulting in the maintenance of a genetically diverse lentivirus population in the host (Goodenow 1989; Meyerhans 1989; Barber 1990; Eigen 1990). It can lead, along with the recombination that occurs during virus replication to the establishment of a more diverse heterogeneic virus population. This variation can be seen within a species-specific feline lentivirus, among a geographic population of individuals, as well as within an individual infected host.

Co-infection.--Co-infection describes the simultaneous infection of a single cell by two or more virus particles. Superinfection is the process by which a cell that has previously been infected by one virus becomes infected with another virus at a later point in time. Recombination after co-infection has been documented to occur both during cross-species transmission of isolates as well as between different viral subtypes within

the same species. Recombination due to co-infection between FIVfca Env variable regions may be relatively frequent during natural infection and may give rise to new clade designations (VandeWoude 2006). Reports describing individuals infected with multiple clade types of FIV, such as FIVfca in a domestic cat and a puma with FIVfco, imply that infected hosts may not be immune to reinfection, or co-infection with different strains of their species-specific virus (Sabino 1994; Robertson 1995; Zhu 1995; Carpenter 1996; Kyaw-Tanner 1996; Carpenter 1998). Evidence of recombination has been reported in Serengeti lions where 40% of those infected were found to be co-infected with two or more FIVple clades (Troyer 2004). However time point data are not available to distinguish whether the dual infections represented a simultaneous co-infection, or a superinfection, in a host.

1.4 Molecular Characterization

The virus has been molecularly characterized in domestic cats into five distinct clades, A, B, C, D, and E, based on the variability in the predicted Env amino acid sequence (Kakinuma 1995; Sodora 1995; Pecoraro 1996; Bachmann 1997; Carpenter 1998; Elder 1998). These clades appear to cluster geographically, with a directly proportional relationship between geographic distance and sequence variation of FIVfca isolates (Bendinelli 1995), although multiple clades have been documented on the same continent. Approximately 80%, or a large majority of viral isolates worldwide are either A or B, however recombinants do occur (Burkhard 2003). B appears to be the oldest subtype, and thus is more host adapted than other subtypes. Clades may exhibit marked differences in pathogenecity, tissue tropism, and clinical disease manifestation (de Monte

2002). Strain virulence is generally related to the clade classification as determined by the sequence of the Env gene (VandeWoude 2006).

Both the lion and puma lentiviruses have been segregated into clades based on sequence data obtained from a fragment of the reverse transcriptase region (RT) of the Pol gene, with FIVple consisting of clades A-F. Note that these clades are distinct from the above named domestic cat clades, as the latter are based on the Env gene (Brown 1994; Troyer 2004; O'Brien 2006). There is significant interspecies sequence divergence, as demonstrated by a comparison among FIVfca, FIVple, and FIVpco, with as much as 30% diversity in the conserved regions of Pol, and even higher diversity in Env and Gag regions (Brown 1994; Langley 1994; Carpenter 1998; Terwee 2005; Troyer 2005). Intraspecies differences are also pronounced, where as much as a 20% divergence exists among Pol of FIVpco and FIVple isolates (Brown 1994; Carpenter 1998; Terwee 2005; Troyer 2005). These findings are in contrast to FIVfca, where clades only differ in their nucleotide sequences by 5-10% across the entire genome (Sodora 1995; Carpenter 1998).

Although geographic clustering of clades is demonstrated with respect to some strains, such as FIVfca and FIVpco (Sodora 1995; Carpenter 1996; Biek 2003), this pattern is not strictly observed for FIVple. For example, the lions of the Serengeti plains region in east Africa were found to be infected with A, B, or C clade viruses, irrespective of geographic location within the Serengeti (Brown 1994; Troyer 2004). Distinct FIVple strains were observed both within members of the same pride as well as within the same individual. However, these PCR products are only partial gene sequences, and there are to date no full FIVple nucleotide sequences available for phylogenetic comparison.

One possible outcome of co-infection could be the recombination between subtypes to form a new viral subtype, as demonstrated in the viral diversity and increased virulence of newly emergent HIV subtypes (Diaz 1995; Robertson 1995; Zhu 1995; Salminen 1997; Wei 1998; Barlow 2001; Holmes 2001). Subtype A was found from lions in both east and southern Africa; subtypes B, C, and F have been detected in east Africa only; and subtypes D and E are found in lions from the Republic of South Africa (RSA) and Botswana, respectively (O'Brien 2006). Thus there are three possibilities for the evolution of FIVple subtypes among lions in geographic isolation: 1) individual hosts were infected with more than one FIVple subtype via viral co-infection or superinfection leading to recombination; 2) primary infection with clonal expansion of one subtype to form a viral quasispecies over time in the host; or 3) a combination of these events. With the exception of the four Kruger National Park lion sequences from Brown *et al* (1994), no sequences have been published or phylogenetically compared for the lion lentiviruses of southern African lions.

Phylogeny.--Feline lentivirus isolates are highly divergent within and among strains, with up to 25% heterogeneity in Pol alone, but are typically monophyletic within a species or family of felids, indicating an inferred common ancestry (Olmsted 1992; Carpenter 1996; Troyer 2005; O'Brien 2006). A study by Brown *et al* (1994) compared RT-Pol partial sequences from FIVple isolates from free-roaming lions in east Africa, southern Africa, and Asia. Phylogenetic analysis from this study revealed several trends, including the monophyly of viral quasispecies within individual lions, suggestive of a clonal expansion of infections, as well as the occurrence of deep ancestral phylogenetic

clades, suggesting the virus likely evolved in allopatric lion populations rather than in another feline species, with transfer to lions (Brown 1994).

It has been suggested that the FIVple divergence from other closely related feline lentiviruses is ancient, and may extend as far back as the radiation of the genus *Panthera* into the great cats (lion, leopard, jaguar, etc), estimated at ~1.6-2.0 million years ago (Wayne 1989; Seidensticker 1991). A comprehensive phylogenetic study by Troyer *et al*, comparing sequences amplified from domestic cats, seven non-domestic feline species, and the spotted hyena found that species lineages tended to be most closely related, and roughly followed a pattern of phylogeography, particularly when comparing amino acid sequences (Troyer 2005).

Phylogenetic analysis of FIVple pol sequences in free-ranging lions revealed the presence of at least six viral subtypes, or clades (A-F) among lions in sub-Saharan Africa (O'Brien 2006). While some of the subtypes have been found thus far in only one geographic region, such as subtypes D in RSA, E in Botswana, C in Serengeti, and F in Kenya, others have been sequenced from multiple geographic populations of free-ranging lions. Among the lions of RSA, subtypes A and D have been detected. Subtype A is shared with the lions of Botswana and Serengeti, whereas subtype D appears to be unique to RSA (O'Brien 2006). All subtypes share a common monophyletic node, suggesting that FIVple infected a common ancestor early in the evolutionary history of the lion. Some viral subtypes then evolved in geographic isolation, while others found in multiple geographic locations may have evolved more recently as a result of lion translocations throughout Africa (O'Brien 2006).

1.5 Transmission

Feline lentiviruses have been isolated from the saliva, blood, serum, plasma, cerebrospinal fluid, milk/colostrums, and genital secretions of infected hosts (Dow 1990; Pedersen 1993; Jordan 1995; O'Neil 1995; Burkhard 1997; Burkhard 2002). Experimental transmission is possible in domestic cats via the oronasal, rectal, vaginal, intravenous, intraperitoneal, and subcutaneous routes, as well as intrauterine and intramammary routes (Bendinelli 1995; O'Neil 1995; Rogers 1998; Allison 2003; Burkhard 2003). The reported seroprevalence for both domestic and exotic feline lentivirus infections increases with sexual maturity, suggesting that horizontal transmission, as with bite wounds, is the primary mode of transmission (Bendinelli 1995; Carpenter 1996; Biek 2003; Luria 2004; Troyer 2005). Intramammary transmission is the most common form of vertical transmission in domestic cats, although maternal antibody is present in FIVfca positive domestic cats up through the first 8-12 weeks of nursing (Burkhard 2003).

Cross-species Transmission.--Cross- species transmission of FIVfca to captive non-domestic felids , presumably from close contact with feral or roaming domestic cats, has been documented in case reports in the scientific literature (VandeWoude 2006). Sequence data supporting cross-species infection with FIVfca has been reported in a captive puma in an Argentinian zoo (Carpenter 1996) as well as in a wild Asian leopard cat (Nishimura 1999), and FIVple has been reported in a captive-born snow leopard and tiger (Troyer 2005). Virus transmission of this nature is rare, and has been typically documented in captive situations where non-domestic felines have an opportunity for prolonged contact or exposure to domestic (feral) cats or other non-domestic feline

species. It should be noted that the typical situation reported thus far in free-ranging felines dictates that most non-domestic felines possess their own unique lentivirus strains. Domestic cats have been experimentally infected with FIVpco and FIVple, yet infection appears to cause little, if any, pathogenicity. An immune response is mounted, however, with cross-reactivity to FIVfca antigens (VandeWoude 2003; Terwee 2005; VandeWoude 2006).

1.6 Epidemiology

Domestic Cat Lentiviruses.--Feline lentiviruses have a broad range of hosts within the family *Felidae* that include domestic cats and non-domestic felids worldwide. It has been estimated that approximately 10% of the world's domestic cats are seropositive, though seroprevalence rates vary according to geographic region (Pedersen 1993; Kanzaki 2004). The lowest seroprevalence rates of 1% are found in central Europe and the United States, with the highest reported seroprevalence of 30% seen in Japan and Australia (Bendinelli 1995). FIVfca seroprevalence in domestic cats is highest among feral animals, adults, and among outdoor males (more aggressive), as well as among domestic cats that are exhibiting clinical signs of illness (Sukura 1992; Pedersen 1993; Courchamp 1994; Peri 1994; Bendinelli 1995; Lin 1995; Carpenter 1998; Courchamp 1998; Winkler 1999; Dorny 2002; Lee 2002; Maruyama 2003; Luria 2004; Kanzaki 2004; Natoli 2005; Troyer 2005).

African Lion Population and Behavior, and its Effect on FIVple Epidemiology.--It has been proposed that the estimated 30,000 remaining free-ranging lions in sub-Saharan Africa today come from a single population of lions, as a result of a population

bottleneck of the modern lion (*ca* 74-203 kyr ago) (Bauer 2003; Yamaguchi 2004). This megapopulation is fragmented into subpopulations that exist throughout sub-Saharan Africa, with little or no natural exchange of individuals (Chardonnet 2002; Bauer 2003). It is estimated that the majority of African lions exist in east and southern Africa (Nowell 2006). The lions of east Africa occupy two contiguous habitats, the Maasai Mara/Serengeti reserve and the Ngorongoro Crater region. The lions of the Ngorongoro have a history of population bottlenecks that reduced the population to as few as 10 individuals (O'Brien 1987; Wildt 1987; O'Brien 1988; Packer 1991). The Serengeti lions, in contrast, are a larger population of approximately 3,000 lions that represent a more genetically diverse population in east Africa (Packer 1986; O'Brien 1987). An estimated 2,000 lions reside in the Kruger National Park (KNP), Republic of South Africa, regarded as one of the largest subpopulations of African lions in southern Africa. The park is RSA's largest wildlife refuge and a critical biodiversity resource, bordering Zimbabwe to the north and Mozambique to the east. KNP stretches roughly 320km from north to south and 65km from east to west.

The social organization of lions typically consists of a pride of related females, their young, and subadult male offspring. Male lions form coalitions and compete with other coalitions for access to prides, and can remain with one pride for months or years at a time, before a new coalition takes over (Sunquist 2002). A study by Spencer *et al.* (1992) of lion lentivirus in southern Africa found the seroprevalence of FIVple among male and female lions to be approximately equal. This finding may be explained by behavior, as prides of females and their offspring allow for social interactive behaviors such as grooming, biting, and play, as well as during mating with adult males, that may

permit virus transmission. Male lions are likely infected through aggressive interactions with other males, whereas females are more likely to become infected through social interactions such as grooming and play between other female pride members, or through sexual interactions with males (Spencer 1992).

Non-domestic Feline Lentiviruses, including FIVple.--Sequence data to support natural, or endemic infection has been reported in both free-ranging and captive non-domestic felids, as well as among spotted and striped hyenas of East Africa (Harrison 2004; Kanzaki 2004; Troyer 2005; VandeWoude 2006). Nineteen out of 35 feline species and 2 members of the family *Hyenidae* examined worldwide have tested seropositive for antibodies to domestic and/or exotic feline lentivirus; 11 species that have been tested were positive using PCR detection, with species-specific strain sequences for at least nine of these species; the remaining 8 species tested were seropositive but did not yield PCR sequences, possibly due to diversity within the primer binding sites, low proviral load, or a combination of these factors (Lutz 1992; Olmsted 1992; Miththapala 1993; Brown 1994; Carpenter 1995; Carpenter 1996; Hofmann-Lehmann 1996; Osofsky 1996; Barr 1997; Posada 1998; Leutenegger 1999; Troyer 2005).

Seroprevalence rates vary greatly among non-domestic free-ranging feline species, with the lions of East and southern Africa showing the highest seroprevalence of all exotic non-domestic free-ranging felid populations tested to date (Olmsted 1992; Brown 1994; Carpenter 1995; Hofmann-Lehmann 1996). A seroprevalence study by Troyer *et al*. (2005) using a three-antigen Western blot screening (domestic cat, puma, and lion lentivirus antigens) found that the seroprevalence of feline

lentivirus among free-ranging non-domestic felids worldwide is highest among African felids, intermediate among non-domestic felids in the Americas, and low among non-domestic felids in Europe and Asia. For example, there is an almost 100% seropositivity for FIVple among the lions of the Serengeti in East Africa, as well as for FIVpco in adult pumas in Wyoming, in the United States (Brown 1994; Biek 2003; Troyer 2004). However FIVple seronegative populations of lions have been reported in free-ranging Asiatic lions, in captive Asiatic lion prides, as well as in Namibian lions of the Etosha pan region (Lutz 1992; Spencer 1992; Brown 1994; VandeWoude 1997; Troyer 2004). FIVpco seroprevalence drops to approximately 20% among pumas in Montana, Florida panthers, and Washington cougars (Evermann 1997; Miller 2006).

A seroprevalence study using a commercial FIV ELISA (IDEXX Laboratories Inc., Westbrook, ME), as well as an ELISA utilizing the p24 antigen of FIV, of southern African lions revealed an 83% seropositivity among the 98 serum samples collected from lions of the Kruger National Park (KNP), RSA, from 1977 to 1991. This same study, however, revealed seronegative results for all 28 serum samples collected from lions in the Etosha National Park, Namibia, from 1989 to 1991, as well as the 7 cheetah, one leopard, and two genet serum samples taken from KNP (Spencer 1992). A later survey reported lentivirus seropositive lions in Namibia's Mahongo Game Reserve (Osofsky 1996). Two KNP leopards tested in 1992 were seropositive (Spencer 1992). A serosurvey of 27 lions in the Hluhluwe-Imfolozi Game Reserve (HIP), South Africa using a commercial FIV ELISA (IDEXX Laboratories Inc., Westbrook, ME) gave no positive results (Spencer 1992; Osofsky 1996). The lion population in HIP, now

approximately 120 individuals, was created from 3 lions translocated from KNP in 1963-64 (Spencer 1992).

Epidemiological analysis of these data revealed no sex preference for seroprevalence among the KNP lions, although there was an increase in seroprevalence with age, from 50% at 6 months old to >80% in those over 6 months. There was also no difference reported according to the geographical distribution of lions sampled in KNP. A study of captive lions in RSA also revealed seropositivity, using the above-mentioned ELISA tests, yet there were no seropositive samples from the other non-domestic felids tested (pumas, leopards, cheetahs, jaguars, tigers, bobcats, servals, caracals; National Zoo Pretoria & Johannesburg Zoo, Johannesburg, RSA) (Spencer 1992). A serological survey of wild felids in Botswana yielded lentivirus positive results using an FIVpco western blot, in eight of 31 lions, three of 18 leopards, and one of four cheetahs (Osofsky 1996).

1.7 Pathogenesis

Clinical Signs and Pathology in Domestic Cats Infected with FIVfca.--FIVfca infection in domestic cats is classified in five stages that parallel manifestations of HIV virus infection in humans. These stages vary widely in their manifestation and length of time from individual to individual, and are known as the: 1) acute; 2) asymptomatic; 3) persistent generalized lymphadenopathy (PGL); 4) AIDS-related complex (ARC); and 5) feline AIDS (FAIDS) disease stages (Pedersen 1993; Bendinelli 1995; Kanzaki 2004).

Acute phase.--The acute phase is recognized by an early peak viremia that typically precedes the onset of clinical signs, and can be accompanied by transient illness

including a generalized lymphadenopathy and fever, a decrease in CD4+ T cells, and the detection of virus in blood lymphocytes, saliva, vaginal secretions, semen, cerebrospinal fluid, and milk/colostrum (Yamamoto 1988; Barlough 1991; Torten 1991; Callanan 1992; George 1993; Matsumura 1993; Beebe 1994). Active virus replication can be seen in the primary tissues for which the virus has a tropism, including the lymphoid tissues, bone marrow, and central nervous system (Pedersen 1993; Kanzaki 2004). The terminal acute phase involves the down regulation of virus replication, as the host's cellular and humoral immunity is activated. This down regulation of virus replication marks the onset of the asymptomatic period, where cell-free virus is found at low levels. In contrast, cell-associated virus is readily detected.

Asymptomatic Phase.--The asymptomatic phase is marked by the absence of obvious clinical disease with a progressive immune dysfunction. This dysfunction is characterized by the continued loss of CD4+ T cells and subsequent inability to produce the cytokine interleukin-2 (IL-2), abnormal cytokine profiles, and an inversion of the CD4/CD8 ratio (Ackley 1990; English 1993; Bendinelli 1995; Diehl 1996; Dean 1996; Willett 1997a). The T-lymphocyte CD4+/CD8+ inversion from a normal value of greater than 2 to a value of less than 2 is due both to a decline in CD4+ cells as well as to an expansion of CD8+ cytotoxic T-cells (Ackley 1990; Pedersen 1993; English 1994; Bendinelli 1995; Dean 1996; O'Neil 1995; Burkhard 1997; Bucci 1998a). The asymptomatic period is not one of virus latency, as the virus has been isolated from the peripheral blood mononuclear cells (PBMC), plasma, and the saliva from apparently healthy (or clinically silent) domestic cats, as is true with lion and other non-domestic feline lentiviruses (Matsumura 1993; VandeWoude 2006).

Persistent Generalized Lymphadenopathy (PGL).-- The next phase, PGL, is characterized by the generalized enlargement of lymph nodes, and can be accompanied by other non-specific signs of illness, such as recurrent fever, anorexia, and weight loss (Yamamoto 1989; Pedersen 1993; Bendinelli 1995). As with the asymptomatic phase, virus can still be isolated from domestic cats in the PGL phase of infection, thus the virus is presumed to be actively replicating (Matteucci 1993).

AIDS Related Complex (ARC).--The progressive immune dysfunction leads to chronic secondary infections of the oral cavity, upper respiratory tract, and gastrointestinal tract, weight loss, generalized lymphadenopathy, and fever, and is known as ARC. The clinical presentation is not severe enough to fulfill the criteria set forth for the feline AIDS complex. It is generally accepted that the ARC is a precursor to the development of FAIDS, thus most cats who present with ARC will eventually progress to the FAIDS stage (Hopper 1989; Ishida 1990; Pedersen 1993).

Feline AIDS (FAIDS).-- The terminal stage, known as feline acquired immune deficiency syndrome (FAIDS), is characterized by a re-emergence of circulating virus with concurrent T cell dysfunction and marked CD4+ cytopenia, with counts at or below 200/µl (Bendinelli 1995; Kanzaki 2004). A study by Hohdatsu *et al*. (2005) suggested that the decreased CD8+ T cell anti-FIV activity observed during the FAIDS stage of lentivirus infection in domestic cats allows for an uncontrolled proviral replication, leading to an increase in proviral DNA copies as the terminal stage of disease progresses. As in human AIDS, multiple secondary opportunistic infections are common, as well as neurologic and neoplastic disorders (Hutson 1991; English 1994; Gabor 2001). Infections are resistant to treatment, and multiple infections are often present in the

individual with FAIDS. Individuals present with a marked emaciation, and a variety of hematological abnormalities, including multiple cytopenias (Bendinelli 1995). The FAIDS stage is considered to be a terminal stage, and the mean survival time can be less than a year (Ishida 1990; Pedersen 1993).

Additional Pathology Not Included in the Stages Above.--In addition to the characteristic T-cell pathology described for lentivirus infection in domestic cats, B-cell abnormalities can be observed and include a polyclonal gammopathy towards non-viral proteins (as early as 6 weeks post-infection), a hypergammaglobulinemia, and more specifically an increase in serum IgG (infection for longer than 24 months) (Ackley 1990; Flynn 1994). Lymph nodes may demonstrate follicular hyperplasia, followed by follicular 'exhaustion' and involution (Bendinelli, 1995). Hematological abnormalities can occur, and include functional alterations in infected cells such as macrophages and lymphocytes (Pedersen 1993; Bendinelli 1995).

Viral Load as an Indicator of Pathogenicity.--Plasma viral loads are the best predictors of disease progression in domestic cats infected with FIVfca, as the disease parallels that seen in HIV infected humans (Diehl 1996; Hirsch 1996; Mellors 1996; Lifson 1997; Nowak 1997; Mellors 1998; Lyles 2000; Pedersen 2001; Goto 2002). Cats with inapparent infection show low viral loads, while those with FAIDS show significant increases in viral load (Lyles 2000), which is closely related to the development of immunodeficiency in feline lentivirus infection (Goto 2002). A study by Hohdatsu *et al.* (2005) suggests that this phenomenon is due to the anti-FIV activity of CD8+ T-cells. This subset of T-cells expands during the earlier stages of disease, including the asymptomatic phase, and helps control the *in vivo* replication of virus in host cells. As

the disease progresses the CD8+ T-cells become depleted. As the number of CD8+ T-cells decline, so does the control of virus replication, hence an increased viral load corresponds to the worsening clinical manifestation of disease.

Clinical Signs and Pathology of Non-Domestic Feline Lentiviruses.--There are several case reports of captive lions exhibiting the clinical signs associated with FIV infection in domestic cats. An 8 year old male African lion with a chronic history of recurrent infections and lymphoma that died in an Italian zoo was diagnosed with an FIV-related infection using antibody detection (western blot using p24 ag) as well as by PCR detection of FIV Gag sequences from lymph node tissue (Poli 1995). The PCR product was not sequenced, however, and it was not determined whether the infection was due to FIVfca or FIVple. A female African lion with inoperable tumors was euthanized at the Zurich zoo, and she, her mate, and offspring were diagnosed with a probable non-domestic FIV (FIVple?) after western blot and ELISA testing for FIV were positive (injection of whole blood from the FIV-positive animals into specific pathogen free cats with no seroconversion or positive culture growth after 11 months) (Lutz 1992). Three captive African lions from the Columbus Zoo in Ohio, U.S.A., were diagnosed with FIVple by virus sequencing and serologic detection, and displayed lymphocyte subset alterations and progressive behavioral, locomotor, and neurologic abnormalities diagnosed as consistent with a lentiviral neuropathy (Brennan 2006). In addition to these case reports, a study by Bull *et al.* (2003) suggested that muscle wasting was a consistent clinical sign observed in FIVple-seropositive African lions from the North Carolina Zoological Park.

Viral Loads in Non-Domestic Feline Lentivirus Infections.--Only two

studies have been published to date that have evaluated the viral loads of non-domestic feline lentiviruses in their natural hosts. One study on plasma viral loads in cougars found that FIVpco-infected cougars maintained viral loads comparable to levels observed in clinically ill FIVfca-infected domestic cats, yet the cougars remained asymptomatic (Blake 2006). In addition it was observed that plasma viremia did not correlate with host age, viral strain, or proviral load in infected cougars. Another study found extremely low proviral loads in all tissues evaluated in an FIVple infected lion, although the lion reportedly died from FIV-associated encephalitis (Brennan, 2006).

1.8 Immune Response to Feline Lentivirus

Domestic Felines.--FIVfca infection elicits a vigorous antibody, T, and B-cell immune response in most domestic cats, however it does not control viral replication and prevent immunodeficiency (Bendinelli 1995; Burkhard 2003). Antibody responses to FIVfca have been detected in naturally infected domestic cats as early as 2-3 weeks after infection, and tend to persist throughout the course of the disease (Yamamoto 1988; Rimmelzwaan 1994; O'Neil 1995; Burkhard 2002). Antibody titers rise over the first 6-8 months of infection, with some cross clade neutralization (Inoshima 1996; Inoshima 1998). FIVfca escapes these antibody mediated clearance mechanisms by changes in antigenicity that result from the high mutation rate of the gene encoding envelope surface (SU) glycoproteins (Siebelink 1993; Siebelink 1995a; Siebelink 1995b; Bendinelli 2001). Both interferon gamma (IFNg+), CD4, and CD8 tumor necrosis factor alpha (TNFa+) T cells respond specifically to FIVfca antigens in domestic cats, leading to a chronic activation of these T cells which eventually die following contact with FIVfca

antigens (Paillot 2005). Perhaps the most well-noted T-lymphocyte alteration is that of the depletion of CD4+ cells concurrent with a relative or absolute CD8+ increase. This leads to a decline in the CD4+/CD8+ ratio from a normal value of two or more, to less than one (English 1994; Bendinelli 1995; Roelke 2006).

Non-domestic Cats.--A depletion of CD4+ T-cells has been documented in both free-ranging and captive lions infected with FIVple (Bull 2003; Roelke 2006). This depletion was profound enough to alter the CD4+/CD8+ ratio, although it was found that the number of CD8+ T-cells was not significantly decreased, as is the case in FIVfca infection in domestic cats. A significant increase of B-cell lymphocytes (CD5- subset) was reported, which is in contrast to domestic cat FIVfca studies. In addition, a suggested signature of FIVple infection is the simultaneous depletion of $CD8+\beta^{high}$ cells and the expansion of $CD8+\beta^{low}$ T-cell subsets (Willett 1993; Bull 2003; Roelke 2006). The $CD8+\beta^{low}$ T-cell expansion is observed in domestic cat FIVfca infection, and these cells are believed to be responsible for the secretion of a soluble factor that is inhibitory for in vitro infection (Bucci 1998a; Bucci 1998b; Shimojima 1998; Gebhard 1999). A study by Terwee *et al.* (2005) suggested that during the early stage of infection with FIVpco and FIVple (as compared to FIVfca) both *in vivo* and *in vitro,* no immune activation of circulating T cells occurs, host cytokine responses are different, and there is no upregulation for the typical markers of infected cell apoptosis seen with FIVfca infection in domestic cats and in humans with HIV. This may help to explain why non-domestic feline lentivirus infections do not appear to be pathogenic for the hosts in which they have evolved. The unique combination of a dramatic decline in CD4+ T-cells, an increase in B-cells, the expansion of $CD8+\beta^{low}$ T-cells, and an increase in CD4-

CD8-CD5+ cells suggests an overall T-cell dyscrasia, as seen in domestic cat FIVfca infection (Roelke 2006). This pattern suggests that lions may be in the process of co-adaptation to the FIVple virus, and thus infection is not entirely non-pathogenic to the host.

The Acute Phase Protein Response.--In addition to the host immune responses discussed above, there is an innate host defense system that is responsible for the host survival during the critical early stages of a response to an insult of an infectious or inflammatory origin, whose purpose is to restore homeostasis and remove the cause of the insult (Ebersole 2000; Eckersall 2000; Evans 1993). This nonspecific and complex series of reactions is referred to as the acute phase response, and involves increases in the concentrations of some plasma proteins, also known as acute phase proteins (APPs) such as haptoglobin (Hp) and alpha-1-acid glycoprotein (AGP) (Kushner 1993; Eckersall 1995). These APPs are glycoproteins that are synthesized primarily by hepatocytes upon stimulation by proinflammatory cytokines such as interleukins and tumor necrosis factor and are released into the bloodstream (Kushner 1993; Yamashita 1994). Lymphocytes and other white blood cells have been shown to produce AGP, whereas Hp was found to be produced in the lung, adipose tissue, spleen, and kidney of some animals (Gahmberg 1978; Dobryszycka 1997; Ebersole 2000; Fournier 2000).

The acute phase response is believed to be a fast response, and may only last a few days, playing a role in the regulation of the immune response as well as in the repair and recovery of damaged tissue (Ceron 2005). However, increases in APPs have also been described in chronic states of disease or inflammation (Horadagoda 1999; Martinez-Subiela 2002). Increased AGP concentrations were found in cats infected with FIVfca

(Duthie 1997). Acute phase protein testing may be used to monitor subclinical disease and to predict possible future clinical disease, although more research is necessary to determine the baseline values found in apparently healthy domestic and non-domestic felines.

1.9 Methods for the detection of feline lentiviruses

General.--Current diagnostic methods for detection of lentivirus infection in domestic and non-domestic felines include viral isolation, immunological tests for virus-specific antibodies or antigens, and molecular tests for viral DNA or RNA (Bendinelli 1995; Crawford 2007).

Viral isolation.--Virus isolation is considered the gold standard for the definitive diagnosis of lentivirus infection. Isolation involves the co-culturing of primary feline blood mononuclear cells (PBMC) of infected cats with the T-lymphocytes of specific-pathogen-free (SPF) cats to allow for virus replication in culture. The cells are initially stimulated with concanavalin A (ConA), and subsequently maintained on human recombinant interleukin-2 (IL-2) (Pedersen 1987; Yamamoto 1988). Virus activity is monitored for Mg2+-dependent reverse transcriptase antigen production and/or FIV-p24 protein, a process that can take up to 7 weeks to detect, particularly in asymptomatic non-domestic felids (Poli 1995; Giannecchini 1996). Infected cats showing clinical signs tend to yield virus positive isolations faster than asymptomatic individuals. As many non-domestic infected felines do not show clinical signs, including lions, it is not the ideal method of diagnostic testing for FIVple due to its labor intensity and the long length of time required for the virus to replicate in culture. The long cultivation periods necessary

to successfully isolate the virus increase the chances for contamination with laboratory or other lentivirus strains, as was reported in a virus isolation attempt from an FIV-seropositive lion (Poli 1995). In addition, lentivirus isolation requires specially equipped laboratories and highly trained personnel, therefore it is primarily used for research purposes (Bendinelli 1995).

Antibody Detection.--As discussed above, host serum antibodies to feline lentivirus infection can develop as early as two weeks after infection, or may take up to 6 months to a year to develop post-infection (Yamamoto 1988; Swango 1991; Bendinelli 1995; Barr 1996). In addition, host maternal antibodies may persist in kittens for six months or longer (Barr 1991). Several rapid, sensitive, and specific enzyme-linked immunosorbent assays (ELISAs) are widely available for the detection of antibodies to FIV in domestic cats (Hartmann 2001). There are presently two commercial FIV ELISA test kits licensed by the USDA for use in domestic cats (SNAP FIV Antibody/FeLV Antigen Combo Test and PetChek FIV Antibody Test Kit, IDEXX Laboratories, Inc., Westbrook, Maine, USA). Previous serological surveys for lentiviruses in non-domestic felids have primarily utilized domestic cat FIVfca capture antigens (Olmsted 1992; Spencer 1992; Brown 1994; Carpenter 1996; Hofmann-Lehmann 1996; Evermann 1997). Although there is some cross-reactivity between FIVple antigens and FIVfca antibodies, it is different with respect to the subtype of FIVfca. Thus FIVfca reagents (particularly monoclonal FIVfca antibodies) may not provide sufficient sensitivity alone for detection of non-domestic cat lentiviruses (VandeWoude 1997). To increase the sensitivity of serological testing, species-specific assays have been developed (Osofsky 1996; VandeWoude 2002; VandeWoude 2003; Troyer 2005), including a Western Blot assay

that utilizes a puma lentivirus (FIVpco) cell lysate as the capture antigen, and an indirect FIVpco synthetic peptide antigen ELISA, with a conserved immunogenic peptide (P237) derived from the Env region of the lentivirus genome (Pancino 1993a; Pancino 1993b; Kania 1997; van Vuuren 2003). These assays have been used for lentivirus testing in lions as well as pumas (Kania 1997; van Vuuren 2003). In addition, the use of a flow cytometry based antibody detection assay has been reported (Biek 2003).

Polymerase Chain Reaction.--There are several polymerase chain reaction (PCR) assays available for the detection of viral RNA or proviral DNA present in the host. These methods include conventional PCR, nested PCR, quantitative competitive PCR (QC-PCR), nucleic acid sequence based amplification (NASBA), and real time PCR (RT-PCR) assays. These assays vary significantly in their ability to detect the virus (or sensitivity), due to the high nucleotide variability of the virus, thus no universal PCR exists for the vast amount of FIV strains circulating among domestic and non-domestic felines worldwide (Hohdatsu 1992; Lawson 1993; Vahlenkamp 1995; Leutenegger 1999; Bisset 2001; Klein 2001; Pedersen 2001; Hohdatsu 2002; Jordan 2002; Lee 2002; Kipar 2004; Crawford 2005; Arjona 2006). Consensus non-domestic feline lentivirus primers cannot be used to identify all non-domestic cat lentiviruses by PCR even when permissive annealing conditions are used (VandeWoude 1997). Thus in order to increase the sensitivity of nucleic acid detection it is necessary to develop PCRs that are specific to the variety of non-domestic viral strains that are identified in captive and free-roaming populations of felids.

Part Two

MATERIALS AND METHODS

2.1 Summary

Whole blood and serum samples were collected opportunistically from free ranging lions in Kruger National Park, Republic of South Africa (RSA), (courtesy of Dewald Keet, chief veterinarian for Kruger National Park in 2004), and from Hlane Royal National Park in Swaziland (courtesy of Dr. Dave Cooper, wildlife veterinarian, Kwa-Zulu Natal, 2004). Whole blood and serum samples were also collected from captive exotic felids at the National Zoological Gardens in Pretoria and captive lions at the Johannesburg Zoo (2003), and from wild and captive born lions at The Lion Park outside Johannesburg (2003-2006). In addition, samples from domestic cats in Johannesburg (2003) were collected courtesy of Friends of the Cat in Johannesburg. Finally, samples from an FIV seropositive (FIVfca SNAP test, IDEXX Laboratories, Westbrook, ME), captive born lion at the St. Louis Zoo, United States (2006) were collected. All RSA samples were collected and stored under the guidance of Dr. Moritz van Vuuren, at the University of Pretoria Faculty of Veterinary Medicine, as part of a research study funded by the South Africa Veterinary Foundation, and the US samples were collected under the guidance of Dr. Kennedy and Dr. Adams at the University of Tennessee, as part of the PhD project.

DNA was extracted from whole blood samples and tested for lion lentivirus using a nested polymerase chain reaction (PCR) assay, followed by 1% agarose gel electrophoresis. Products from all positive PCR reactions were purified and submitted for nucleotide sequencing at the Molecular Biology Resource Facility (MBRF) DNA sequencing laboratory at the University of Tennessee. Sequences were confirmed as lion lentivirus by Basic Local Alignment Search Tool analysis

(http://www.ncbi.nlm.nih.gov/BLAST/), and were analyzed phylogenetically using the DNAstar lasergene software program (DNASTAR Inc., Madison, WI).

Serum samples were tested for antibodies to domestic feline lentivirus antigens using a SNAP Combo FeLV Ag/FIV Antibody Test (FIVfca ELISA) (IDEXX Laboratories, Westbrook, ME), as well as an ELISA for antibodies to FIVpco synthetic Env peptide antigen (FIVpco ELISA). Serum samples were additionally tested for feline haptoglobins (Hp) and feline alpha-one acid glycoproteins (AGP) by radial immunodiffusion. Finally, data collected from lions in the Kruger National Park, RSA, were analyzed in conjunction with the molecular and serological lentivirus data as part of a descriptive epidemiological study on lion lentivirus in a free-ranging population of lions.

2.2 Sample Collection & Storage

The samples available for evaluation by Dr. Adams included: Epidemiological data from 77 lions in the Kruger National Park, for which 73 whole blood and 63 serum samples were taken; whole blood and serum samples from 7 lions in The Lion Park, Johannesburg; 11 banked frozen cell culture samples from KNP Mycobacterium tuberculosis positive lions (virus isolation was previously attempted as part of a study by Dr. van Vuuren, and cell culture fluid containing the cell pellet was frozen for further research); 8 cell culture samples from lions in the Hlane Game Reserve; cell culture samples from 3 lions in the Johannesburg Zoo; cell culture samples from 4 tigers and 3 leopards in the Pretoria Zoo; cell culture samples from 7 domestic cats in Johannesburg; and whole blood and serum from one lion in the St. Louis Zoo, United States.

All whole blood, serum, and cell culture samples were stored at -80 degrees Celsius (C) until the time of processing and testing. All RSA samples were stored at the University of Pretoria Veterinary Faculty, Department of Veterinary Tropical Disease, and US samples were stored at the University of Tennessee. All FIVpco ELISA assays were performed by Mrs. Sandra Prinsloo, Head Serology Technician, at the University of Pretoria, under the guidance of Dr. Moritz van Vuuren (2004-2006). All whole blood extractions, PCR testing, FIVfca ELISA testing, AGP testing, and Hp testing was performed by Dr. Adams at the University of Pretoria and the University of Tennessee (2006).

2.3 DNA Extraction

DNA was extracted from whole blood and cell culture samples using the QIAGEN DNeasy Blood and Tissue Extraction kit (Invitrogen Corp., Carlsbad, CA), resulting in 100 microliters of purified DNA. DNA extraction was performed by Dr. Adams in the extraction laboratory of the University of Pretoria Veterinary Faculty Department of Tropical Diseases, according to the manufacturer's instructions (Protocol: Purification of total DNA from animal blood, Spin-Column,QIAGEN DNeasy Blood & Tissue Handbook, 2006, p. 25). Briefly, 100 microliters of whole blood sample was mixed with 20 microliters of proteinase K(>600mAU/ml) and 100 microliters of 1X phosphate buffered saline (PBS; 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) and 200 microliters of Buffer AL (Lysis Buffer) and incubated at 56 C for 10 minutes. Two hundred microliters of 100 % ethanol was added and the mixture was placed into a spin column and centrifuged, followed by two washes with Buffer AW1 and Buffer

AW2, respectively, centrifugation, with a final elution in Buffer AE (10mM Tris, pH 9.0; 0.5 mM EDTA) of 100 microliters. All DNA extraction products were held in storage at -80C, and were placed on ice and shipped to the United States with Dr. Adams for PCR testing and sequencing at the University of Tennessee.

2.4 Polymerase Chain Reaction

A nested conventional PCR was performed on the purified DNA, using a protocol developed by Troyer *et al.* for FIVple (2004; 2005) and optimized by Dr. Adams for use in testing southern African lions. Primers (shown in Table 2.1) were derived from GenBank published sequence information for FIVfca (M25381 & U11820), FIVpco (U03982), and FIVoma (U56928) by Troyer *et al.* (2004). The degenerate primers were developed to detect viral DNA from the RT-Pol (or reverse transcription region of Pol) region of the lentivirus genome, resulting in a nested 576 BP product (including nested primers). The 5' and 3' outer oligonucleotide sequences were TGGCCWYTAWCWAATGAAAARATWGAAGC (referred to as P1F) and GTAATTTRTCTTCHGGNGTYTCAAATCCCC (referred to as P2R), respectively. The nested 5' and 3' oligonucleotides were TGAAAARATWGAAGCHTTAACAGAMATAG (referred to as P2F) and GTAATTTRTCTTCHGGNGTYTCAAATCCCC (referred to as P1R), respectively. All primers were synthesized by Sigma Genosys (Sigma Corporation, The Woodlands, TX), and were reconstituted to a 20 picomole working solution.

Table 2.1. List of primers used in PCR testing for FIVple. Nucleotide sequences as well as the genome position are provided, with respect to the FIVfca Petaluma strain (Genbank #323933).

Primer	Sequence	5' Position
G1F	**AGTKGGCGCSMGAACWSGWCYTGA**	**355**
G2F	**AATGTAWCTACAGGACGAGAA**	**754**
P1F	**TGGCCWYTAWCWAATGAAAARATWGAAGC**	**2407**
P2F	**TGAAAARATWGAAGCHTTAACAGAMATAG**	**2421**
P3F	**TAGAYTGGYHCCWGGWCATAAA**	**3931**
P4F	**GRGARGAYGCAGGATATGA**	**4030**
G1R	**GCCATRTCWGTWGRTGTTAA**	**1125**
G2R	**TCCTARYCCWTCTCTWGCYTTYTCCA**	**1187**
P1R	**GTAATTTRTCTTCHGGNGTYTCAAATCCCC**	**2997**
P2R	**GTATTYTCTGCYTTTTTCTTYTGTCTA**	**3427**
P3R	**TTRSGCSADYARRTCATATCCTGC**	**4061**
P4R	**ATTSKAAARTTTGGKCCATTATC**	**4760**
P5R	**TGGTCCATTATCWGWTTGTA**	**4767**

Key:
The standard codes for degenerate bases are as follows:
A = Adenosine;
C = Cytosine;
G = Guanosine;
T = Thymidine;
B = C,G, or T;
D = A,G, or T;
H = A,C, or T;
V = A,C, or G;
R = A or G (puRine);
Y = C or T (pYrimidine);
K = G or T (Keto);
M = A or C (aMino);
S = G or C (Strong -3H bonds);
W = A or T (Weak - 2H bonds);
N = aNy base.

The successful nested primer set (Figure 2.1) was used to generate DNA using Platinum® PCR SuperMix (containing 22 U/ml complexed recombinant *Taq* DNA polymerase with Platinum® *Taq* Antibody, 22 mM Tris-HCl at pH 8.4, 55 mM KCl, 1.65 mM MgCl2, 220 micromolar dGTP, dATP, dTTP, and dCTP, and stabilizers), (Invitrogen Corporation, Carlsbad, CA). The 54 microliter total volume first round PCR reaction mixture consisted of 45 microliters PCR SuperMix, 2 microliters (20 pmol) of primer P1F, 2 microliters (20 pmol) of primer P2R, and 5 microliters of sample template. The cycling conditions for the first round PCR reaction consisted of a pre-denaturing step of 3 minutes at 94C, 45 cycles of 30 sec at 94C (denaturing), 30 sec at 52C (annealing), and 45 sec at 72C (extension), with a final extension for 10 minutes at 72C. Three microliters of the resultant PCR product from round one were added to a nested PCR reaction mixture consisting of 45 microliters of PCR SuperMix, one microliter of primer P2F, one microliter of primer P1R, for a total volume per sample of 50 microliters. The cycling conditions for the nested reactions were the same as for the first round PCR reaction. All reactions were carried out in an Eppendorf Mastercycler® personal PCR thermocycler (Eppendorf, Westbury, NY).

In addition to the nested primers P1F, P2R, P2F, and P1R, PCR was performed using all primer sets listed in Tables 2.1 and 2.2, in accordance with the protocol described above. These primers were unsuccessful in producing any PCR products or positive controls for our samples.

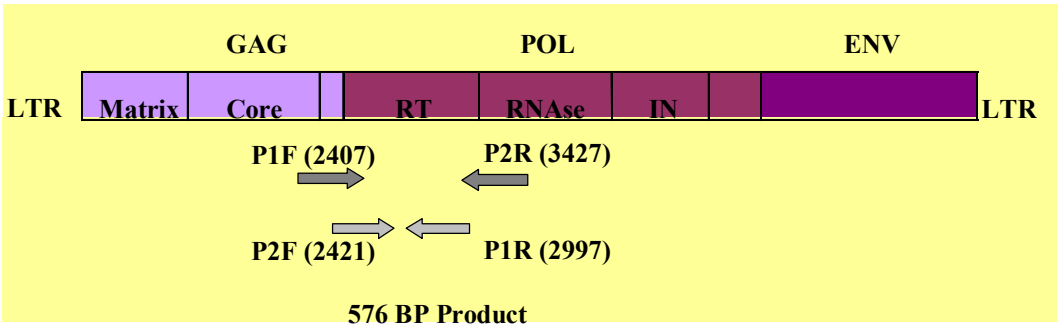


Figure 2.1. Schematic representation of the FIV genome, including the successful first and second round primers used and their nucleotide position within the RT-Pol region of the genome.

Table 2.2. Primer combinations attempted for first and second round (nested) PCR to detect FIVple. Genomic region and product size are indicated.

1st Round Primers	2nd Round Primers	Gene Region	Product Size
G1F-G1R	**None**	**LTR/GAG**	**770 bp**
G1F-G2R	**G2F-G1R**	**GAG**	**444 bp**
P1F-P2R	**P2F-P1R**	**POL-RT**	**576 bp**
P3F-P3R	**None**	**POL-RNASE**	**118 bp**
P3F-P5R	**P4F-P4R**	**POL-RNASE**	**730 bp**

2.5 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed on all PCR samples to visualize amplification products. Ten microliters of each amplified DNA product was combined with 2 microliters of 6X loading buffer and was loaded into a 1% agarose gel stained with ethidium bromide in 1X TBE buffer (Tris 0.9 M, Borate 0.02 M, and EDTA 0.02 M, pH 8.3). A 1 Kb DNA ladder (exACTGene 100bp DNA Ladder, Fisher Scientific International, Inc., Pittsburgh, PA) was used for size estimation of all samples. The agarose gel with loaded samples was then placed in a 1X TBE buffer bath and was electrophoresed for 25 minutes at 125 volts, after which time the gel was transilluminated to reveal DNA products.

2.6 PCR DNA Purification

DNA amplicons were extracted from agarose gel using the QIAGEN QIAquick gel extraction kit® (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions (QIAGEN QIAquick gel extraction kit protocol, QIAquick Spin Handbook, 2002, p. 23). Briefly, DNA fragments were excised from the agarose gel, placed into a microcentrifuge tube with buffer and incubated to dissolve the gel slices. Isopropanol was then added to the samples to increase the yield of the DNA fragments. This mixture was then placed in a spin column and centrifuged to bind DNA to the QIAquick membrane. After washing with buffer, the DNA was eluted with sterile water for a final elution volume of 28 microliters. All positive PCR products were submitted for molecular sequencing at the University of Tennessee Molecular Biology

Resource Facility (MBRF) DNA sequencing laboratory. The primers used for sequencing were P2F and P1R, at a 5 pmol solution.

2.7 Sequence Analysis

Sequences were confirmed as FIVple using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) software program (http://www.ncbi.nlm.nih.gov/) using a search program for highly similar sequences (megablast). Phylogenetic analysis was performed on all sequences obtained using DNAstar lasergene software (http://www.dnastar.com/).

2.8 FIVpco ELISA

Ninety-nine serum samples were tested for cross-reactive antibodies with an indirect enzyme-linked immunosorbent assay (ELISA) using a synthetic FIVpco peptide (CPFKDICQL, AA 610-618, GenBank U03983) located on the envelope (Env) glycoprotein, corresponding to the peptide P237 of FIVfca (CNQNQFFCK). The FIVpco ELISA was developed by Dr. Stephen Kania at the University of Tennessee. Prior research with this ELISA demonstrated cross-reactivity to lion IgG antibodies (Kania 1997; van Vuuren 2003).

Briefly, the peptide was diluted in PBS at 1:1000 (10µg/ml was diluted 10µl Ag + 10ml PBS), added to a 96 well microplate (Immulon, Thermo Lab Systems, MA) at 100µl/well, and incubated at between 2-8C overnight. Plates were then washed four times with PBS-$Tween_{20}$ (0.5ml polyoxyethylene sorbitan monolaurate / liter PBS = 0.05% $Tween_{20}$), and blocked by adding 200µl of PBS-$Tween_{20}$ and waiting for one hour.

Serum was diluted to 1:25 in PBS-Tween$_{20}$ (i.e. 40µl serum + 960µl PBS-T), and 100µl of diluted serum was added to the appropriate wells. Plates were sealed and incubated for one hour at 37C, followed by four washes with PBS-T as described above. Next, 100µl of anti-cat IgG HRPO conjugate was added to all test wells (Dilute 1:2000, i.e. 5µl + 10µl PBS-T), followed by sealing, incubation, and four washes as described above. After washing, 100µl of 2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) substrate was added to each well, and plates were placed in the dark for ± 25 mins / until good color development was achieved, followed by reading at 405nm (Bio-Tek EL808 Microplate Reader, Bio-Tek, VT). Cut-off values were equal to double the negative value, and positive values were considered to be greater than or equal to the cut-off value (Kania 1997).

2.9 FIVfca ELISA

Thirty-three serum samples were tested for cross reactive antibody against FIVfca *gag* p24 protein antigens (Mermer 1992), with an ELISA known as the SNAP Combo FeLV Ag/FIV Antibody Test (IDEXX Laboratories, Westbrook, ME), according to the manufacturer's instructions. Samples were selected randomly using a random number generator. Domestic cat FIV *gag* products are most likely to be highly immunogenic yet conserved among different feline lentivirus isolates, including FIVple isolates (Dowbenko 1985; Coffin 1986). Briefly, serum samples (if positive, containing antibodies to FIV) were mixed with inactivated FIV antigen-enzyme conjugate (provided) and placed into the sample well of the SNAP device, which contained FIVfca antigens in the matrix of the well. Test results were interpreted after 10 minutes. A

positive test result was indicated by a color development in the FIV antibody sample spot, according to the manufacturer's package insert, along with the appropriate color development in the positive control spot.

2.10 Serum Haptoglobins (Hp)

Serum samples were measured for feline haptoglobins in 52 lion samples from Kruger National Park, using a domestic feline haptoglobin measurement kit (Cardiotech services, Inc, Louisville, KY). Briefly, each serum sample was diluted 5-fold and placed into a test well. Test plates were incubated at 37C for 24 to 48 hours. Samples diffused radially from the well into an agar gel plate containing antiserum to domestic feline Hp (rabbit serum), forming a visible ring of precipitation directly proportional to the concentration of Hp in the diluted serum samples. Ring diameter measurements allowed for the calculation of Hp concentration, as compared with two known standard test solutions provided with the kit. The Hp concentrations were plotted on semi-logarithmic graphs.

2.11 Serum Alpha-one acid glycoproteins (AGP)

Serum samples were measured for feline alpha-one acid glycoproteins (AGP) in 52 lion samples from Kruger National Park, using a domestic feline AGP measurement kit (Cardiotech services, Inc, Louisville, KY). Each serum sample was placed (full strength) into an individual test well. Test plates were incubated at 37 degrees Celsius for 24 to 48 hours. Samples diffused radially from the well into an agar gel plate containing antiserum to domestic feline AGP, forming a visible ring of precipitation directly

proportional to the concentration of AGP in the serum samples. Ring diameter measurements allowed for the calculation of AGP concentration, as compared with two known standard test solutions provided with the kit. The AGP concentrations were plotted on semi-logarithmic graphs.

2.12 Epidemiological Data

Epidemiological data were collected from 77 of the Kruger National Park lions at the time of blood collection. Data gathered included: pride name as designated by KNP Research Staff; approximate location in KNP as designated by recognizable landmarks; animal's identification number (microchip ID number implanted upon first immobilization in each KNP lion; numbers designated by KNP chief veterinarian, Dewald Keet); sex; age as estimated by tooth morphology (Dewald Keet, personal communication); body weight; and body condition score (1-5). A description of the body condition scoring used as part of the data sheet is given below, provided by Dewald Keet: *Condition Scoring of Live Lions in the Kruger National Park.--*

5. Excellent: Hindquarters well rounded and no ribs showing; general appearance in relation to posture and coat sheen excellent.

4. Good: Hindquarters rounded, ribs showing slightly.

3. Fair: Hindquarters angular in appearance and ribs well defined.

2. Poor: Pelvic bones and pelvic-femoral joint prominent and ribs protruding. Tail root is sunken in. The dorsal spine of the vertebrae becomes apparent.

1. Very Poor: Skeletal details clearly visible and general appearance, posture, and coat condition deteriorated. The dorsal and lateral processes of the vertebrae are clearly visible.

Epidemiological data were correlated with the results from ELISA, PCR, and Acute Phase Protein testing for each animal for which all data were available.

Part Three

RESULTS

3.1 Samples

A total of 77 lions were anesthetized and sampled in the Kruger National Park (KNP) during a 2004 census of the lions, and epidemiological information was taken on each lion. Of the 77 lions tested during the census, a total of 73 whole blood samples and 63 serum samples were obtained for lentivirus testing. Of the 63 serum samples, all were tested with the FIVpco ELISA, however only 52 samples were banked in frozen storage, and thus available for further testing (FIVfca ELISA, APPs). In addition to these samples, 11 samples of banked cell culture used for virus isolation from whole blood from KNP lions were used for testing. However no whole blood, serum, or epidemiological information was available for the latter samples. All 11 cell culture samples were from lions that had tested positive for Mycobacterium tuberculosis (Tb). The Tb status of the 77 lions for which epidemiological information was provided was unknown. The 11 banked cell culture samples from KNP Tb lions will be referred to as the KNP-Tb subset, and the 77 KNP lions for which epidemiological information was provided as the KNP-Epi subset. Because geographic location was recorded for each animal, the KNP-Epi subset can be further subdivided into the KNP-Epi North (KNP-EPI-N) and KNP-Epi South (KNP-EPI-S) lions, and then subdivided by pride. The remaining banked cell culture samples provided for testing in RSA came from: 8 lions in the Hlane Game Reserve; 7 lions in The Lion Park (LP), Johannesburg; 3 lions in the Johannesburg Zoo (Jhb); 4 tigers and 3 leopards in the Pretoria Zoo (Pta); and 7 domestic cats in Johannesburg (DC). Finally, whole blood and serum was provided from one lion in the St. Louis Zoo (StL), United States. All samples collected and tested are listed in Table 3.1.

Table 3.1. Blood and serum samples and selected epidemiological data collected free-ranging lions and other felids in South Africa from lentivirus testing ,2003-2006.

Sample Subset	Epidemiological Data Recorded	Whole Blood Available for testing	Serum Available for testing	Cell Culture Fluids Available for Testing	IDEXX SNAP ELISA (FIVfca)	FIVpco ELISA	PCR	Sequencing	APP Tested
KNP-Epi-N	Yes (26)	Yes* (23)	No	No	No	Yes* (22)	Yes* (23)	Yes* (9)	No
KNP-Epi-S	Yes (51)	Yes* (50)	Yes* (52)	No	Yes* (23)	Yes* (41)	Yes* (50)	Yes* (35)	Yes (52)
KNP-Tb	No	No	No	Yes (11)	No	Yes (11)	Yes (11)	No	No
Hlane	No	No	No	Yes (8)	No	Yes (8)	Yes (8)	No	No
Pta Zoo	No	No	No	Yes (7)	No	Yes (7)	Yes (7)	Yes* (1)	No

Table 3.1 continued: Blood and serum samples and selected epidemiological data collected free-ranging lions and other felids in South Africa from lentivirus testing ,2003-2006.

Jhb Zoo	**No**	**No**	**No**	**Yes (3)**	**No**	**Yes (3)**	**Yes (3)**	**No**	**No**
LP	No	Yes (7)	Yes (7)	No	Yes* (2)	Yes (7)	Yes (7)	No	No
DC	No	No	No	Yes (7)	Yes (7)	No	Yes (7)	Yes* (6)	No
StL Lion	No	Yes (1)	Yes (1)	No	Yes (1)	No	Yes (1)	Yes (1)	No
Total samples tested	77	81	60	36	33	99	117	52	52

*Indicates a portion; not all samples in the subset were tested or have results available.
**Column one describes the sample identification according to geographic location. Columns 2 through 5 indicate what data/samples were collected. Columns 6 through 10 indicate which tests were performed. Numbers in parentheses indicate the number of samples tested.

Table 3.1 continued: Blood and serum samples and selected epidemiological data collected free-ranging lions and other felids in South Africa from lentivirus testing ,2003-2006.

Key:
KNP Epi-N: Lions in the Kruger National Park, Republic of South Africa, North section.
KNP Epi-S: Lions in the Kruger National Park, Republic of South Africa, South section.
KNP-Tb: Lions from Kruger National Park, Republic of South Africa, who were previously determined to be tuberculosis positive.
Hlane: Lions from Hlane Game Reserve, Swaziland.
Pta Zoo: Leopards and tigers in the Pretoria Zoo, Republic of South Africa.
Jhb Zoo: Lions in the Johannesburg Zoo, Republic of South Africa.
LP: Lions in The Lion Park, Johannesburg, Republic of South Africa.
DC: Domestic cats in Johannesburg, Republic of South Africa.
StL Lion: Lion at the St. Louis Zoo, U.S.A.

3.2 Polymerase Chain Reaction

Of the 117 samples tested with PCR, 52 were positive and 65 were negative for lion lentivirus. There were no positives from the cell culture samples of the KNP-Tb subset. Forty-four samples from KNP-Epi lions were positive and 29 were negative out of 73 samples. Of the KNP-Epi samples, 9 positives were from the 23 KNP North lions sampled, and 35 positives were from the 50 KNP South lions tested. One Hlane lion tested was PCR positive out of the 8 lions sampled. None of the 7 lions tested from The Lion Park were positive. One tiger from the Pretoria Zoo tested positive, out of four tigers tested, and all three of the Pretoria Zoo leopards tested negative, as well as the three lions from the Johannesburg Zoo. Five of the seven domestic cat samples from Johannesburg tested positive by PCR. Finally, the St. Louis lion tested positive for lion lentivirus by PCR. Figure 3.1 displays the positive bands illuminated after gel electrophoresis. Figure 3.2 displays the PCR results by subset sampled.

3.3 Sequencing

A total of 34 sequences from the RT-Pol region of the lion lentivirus genome were obtained out of the 52 PCR positives that were submitted for sequencing analysis. Of these 34 sequences, 21 were obtained from lions in the south of Kruger National Park, 6 sequences from lions in the north of Kruger National Park, 5 sequences were from domestic cats in Johannesburg, one sequence came from a tiger at the Pretoria Zoo, and one sequence came from a lion at the St. Louis Zoo. Eighteen of 52 PCR positives did not produce a viable sequence. Sequences were placed in Genbank under EF667006-EF667017; EF667019-EF667020; and EF667022-EF667041.

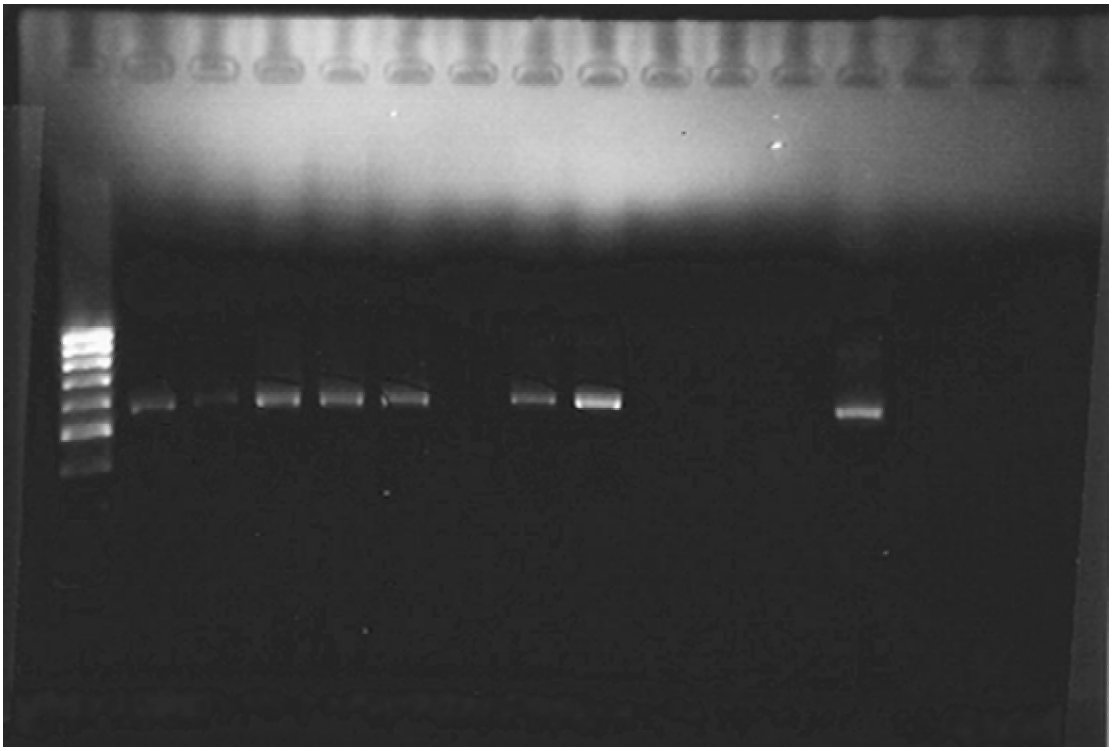


Figure 3.1. 576 band size Nested PCR products produced from P2F-P1R primers, and visualized on a 1% agarose gel containing ethidium bromide. A 1 Kb ladder was used as a standard.

Key:
Lane 1: 100 Kb Ladder
Lane 2: Sample 1; positive.
Lane 3: Sample 2; positive.
Lane 4: Sample 3; positive.
Lane 5: Sample 4; positive.
Lane 6: Sample 5; positive.
Lane 7: Sample 6; negative.
Lane 8: Sample 7; positive.
Lane 9: Sample 8; positive.
Lane 10: Sample 9; negative.
Lane 11: Sample 10; negative.
Lane 12: Blank.
Lane 13: Positive Control Sample.
Lane 14: Negative Control Sample.

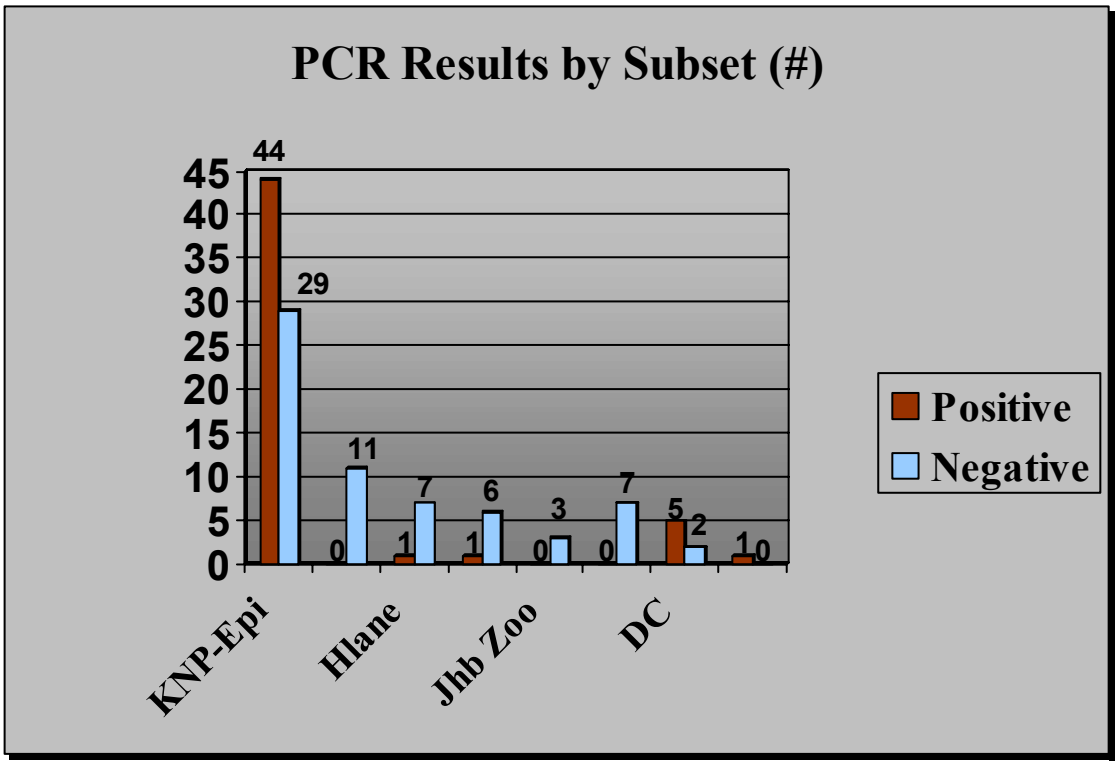


Figure 3.2. Nested PCR positive and negative results for each felid blood sample collected between 2003-2006, according to sample subset (location).

Key:
KNP Epi-N: Lions in the Kruger National Park, Republic of South Africa, North section, who were not tuberculosis tested.
KNP Epi-S: Lions in the Kruger National Park, Republic of South Africa, South section, who were not tuberculosis tested.
KNP-Tb: Lions from Kruger National Park, Republic of South Africa, who were previously determined to be tuberculosis positive.
Hlane: Lions from Hlane Game Reserve, Swaziland.
Pta Zoo: Leopards and tigers in the Pretoria Zoo, Republic of South Africa.
Jhb Zoo: Lions in the Johannesburg Zoo, Republic of South Africa.
LP: Lions in The Lion Park, Johannesburg, Republic of South Africa.
DC: Domestic cats in Johannesburg, Republic of South Africa.
StL Lion: Lion at the St. Louis Zoo, U.S.A.

Sequences were checked for homology using BLAST technology. Appendix A displays the closest homologous sequence from GenBank for each sequence obtained. All sequences amplified from domestic cat hosts (DC subset) were homologous to FIVfca. All KNP sequences amplified from lions, as well as from the St. Louis lion, were homologous to FIVple. The sequence amplified from a tiger at the Pretoria Zoo was homologous to FIVfca, indicating a cross-species infection.

3.4 Phylogenetics

A series of phylogenetic trees were constructed for the nucleotide sequences amplified from PCR, and can be viewed in Figures 3.3 and 3.4. Figure 3.3 displays the FIVple sequences for KNP-Epi north and south lions. In general, clades were formed based on geographic location of north or south within the park, and were supported by high bootstrap values. There was some overlap between north and south clades, and overall the KNP sequences shared an ancestral node supported by a high bootstrap value.

Figure 3.4 shows a phylogenetic tree of some KNP sequences along with sequences from FIVple subtypes A-F, originally amplified from East African lions. Subtype A formed a clade with south KNP sequences; D and F formed a clade with a mix of south and north KNP sequences; subtypes B and C form a clade separate from all other sequences, showing a more distant phylogenetic relationship; and subtype E formed a clade with KNP north sequences.

All amplified sequences are displayed in Appendix B, including those from KNP lions, domestic cats in Johannesburg, a tiger from the Pretoria Zoo, and the St. Louis Zoo

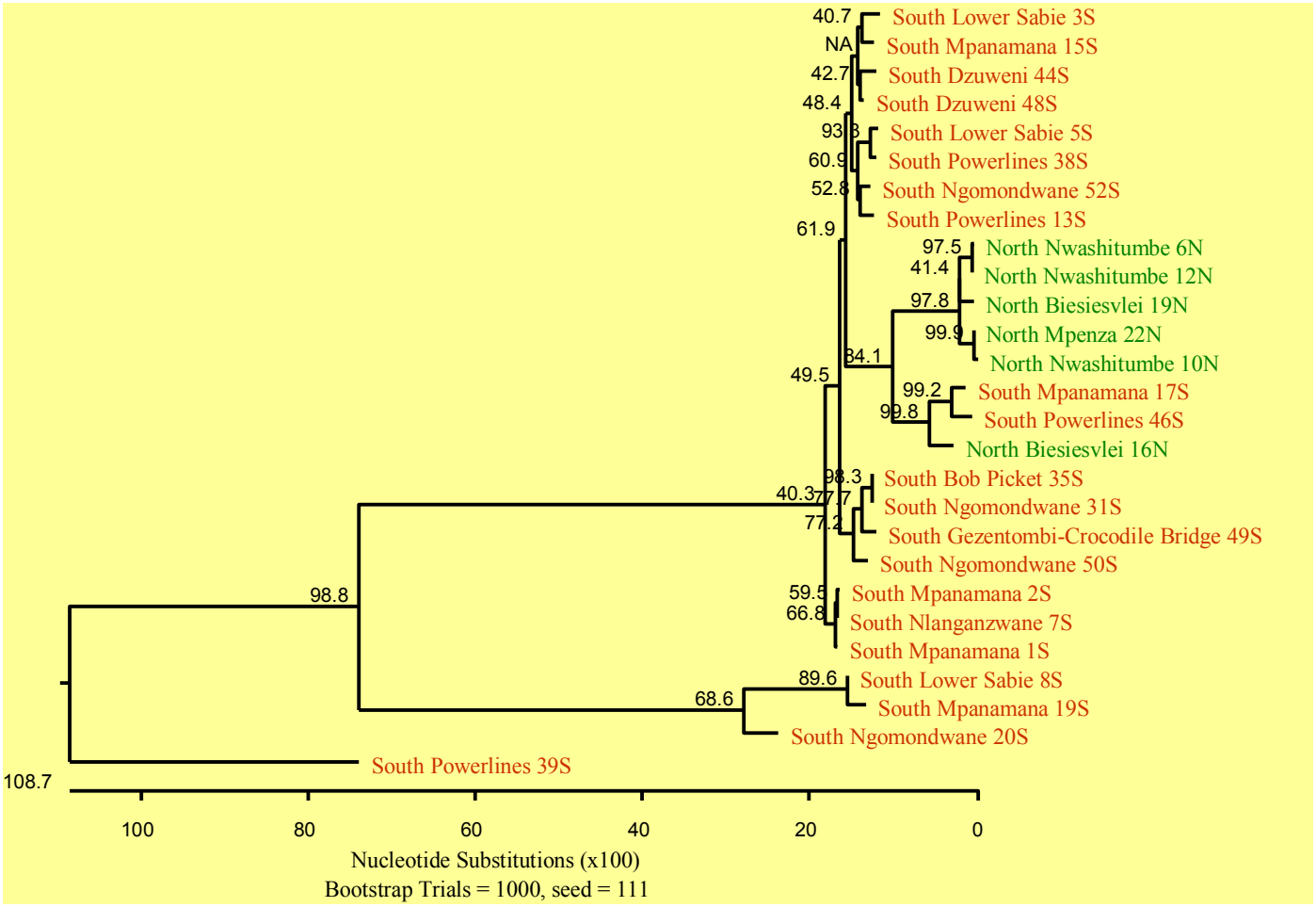


Figure 3.3. RT-pol phylogenetic tree of sequences from lions in the north and south sections of Kruger National Park, Republic of South Africa.

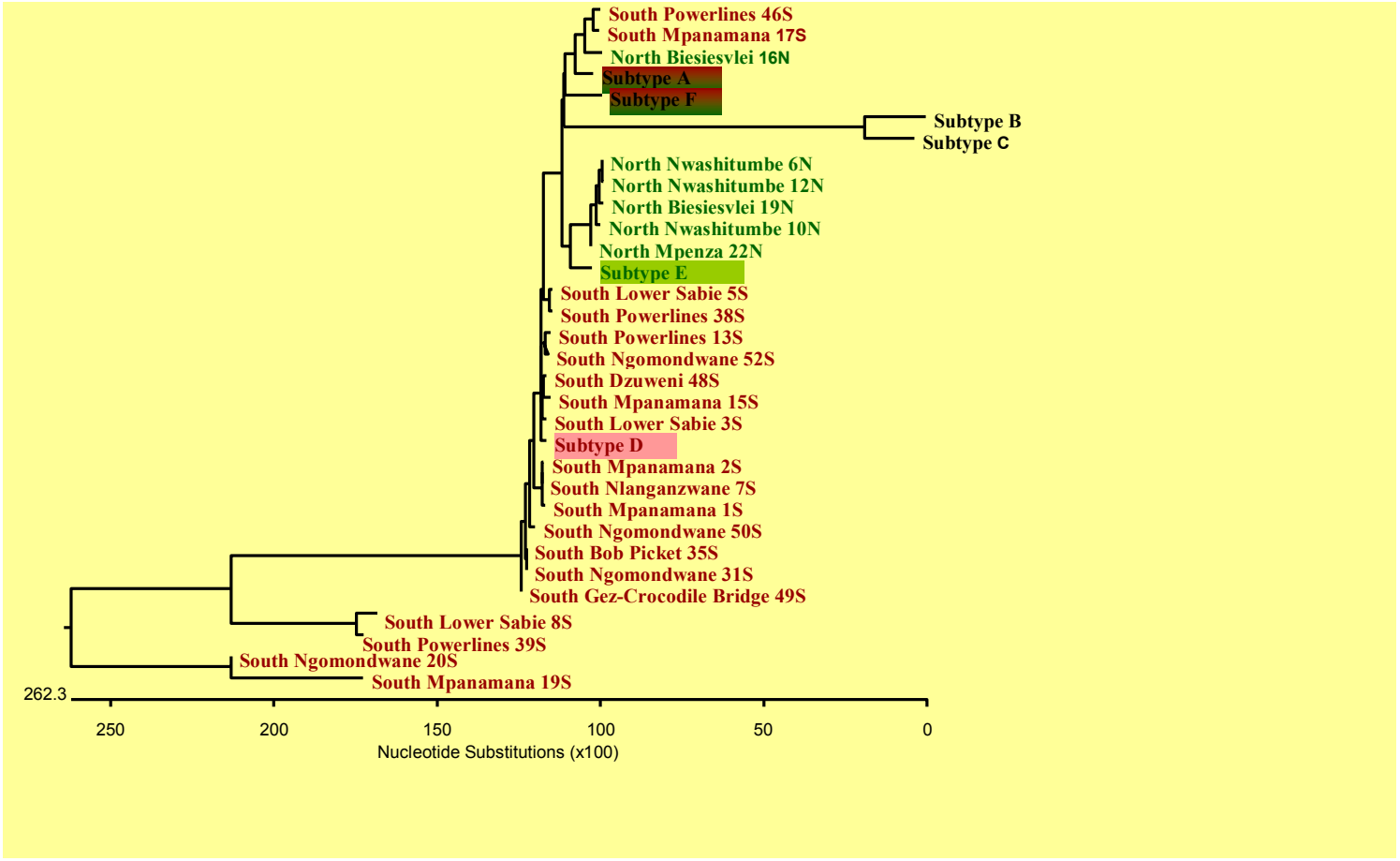


Figure 3.4. RT-pol phylogenetic tree of sequences from lions in Kruger National Park, Republic of South Africa. Reference RT-pol NT sequences representing FIVple subtypes A-F, sequenced from East African lions, have been added for comparison.

*East Africa subtypes: A (Genbank AY549255); B (Genbank AY552669); C (Genbank AY552683); D (Genbank AY878214); E (Genbank AY878235); and F (Genbank AY878220) sequences from Troyer (2004).

lion. The domestic cat FIVfca sequences formed a clade that included the tiger FIVfca sequence, and the St. Louis Zoo lion sequence formed a clade with KNP south isolates.

3.5 FIVpco ELISA

Fifty-two samples tested positive and 47 tested negative to the FIVpco ELISA, out of the 99 samples tested. A total of 38 out of 63 KNP-Epi lion and samples were positive: 11 positives were from KNP-Epi-N lions; 27 positives were from KNP-Epi-S lions; and the remaining 11 positives were from KNP-Tb lions. The remaining test-positive animals included all seven domestic cats from Johannesburg, and 3 out of 8 Hlane lions. The results from FIVpco ELISA testing are displayed in Figure 3.5.

3.6 FIVfca ELISA

Twenty-seven out of 33 serum samples tested positive using the FIVfca ELISA, with the remaining six samples negative. Of these 33 samples, 19 positives and four negatives came from KNP-Epi-S lions. All seven domestic cats tested positive to the FIVfca ELISA. The remaining positive sample came from a lion from the St. Louis Zoo, and the final two negatives came from lions at The Lion Park in Johannesburg. No FIVfca ELISAs were performed on the KNP-Epi-N lions, as no serum samples were available for testing from KNP-Epi-N lions. The results from FIVfca ELISA testing are displayed in Figure 3.6.

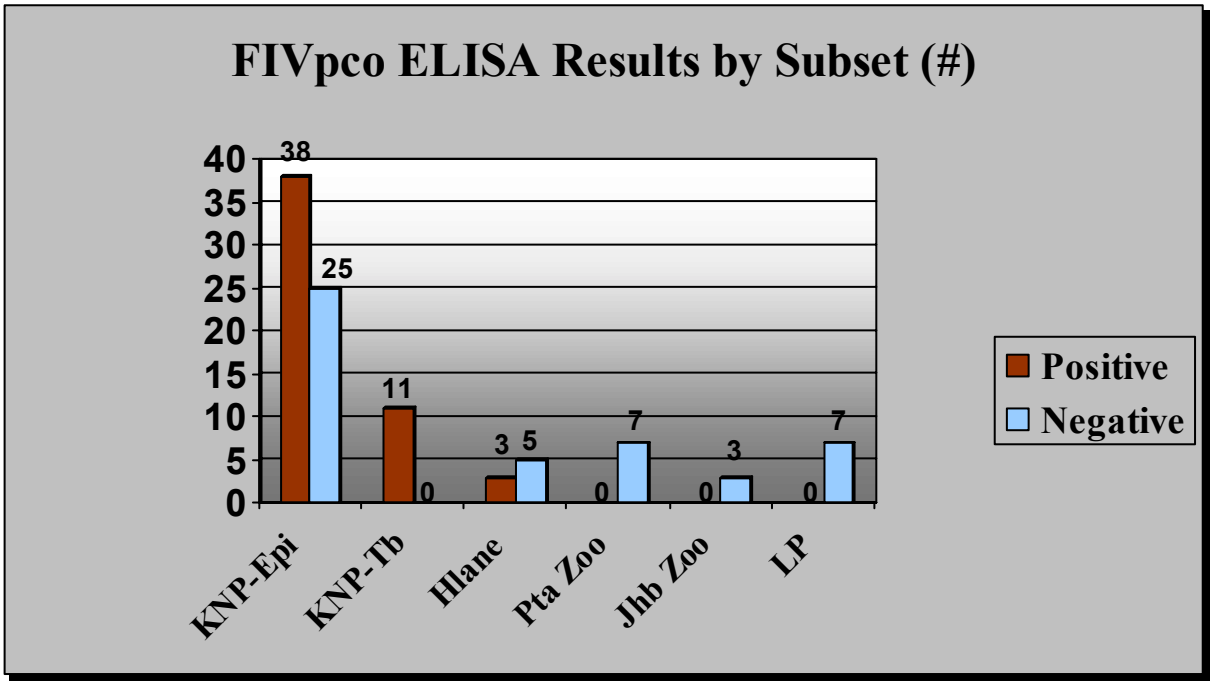


Figure 3.5. FIVpco ELISA results from felid serum samples collected between 2003-2006, according to sample subset (location).

Key:
KNP Epi: Lions in the Kruger National Park, Republic of South Africa who were not tuberculosis tested.
KNP-Tb: Lions from Kruger National Park, Republic of South Africa, who were previously determined to be tuberculosis positive.
Hlane: Lions from Hlane Game Reserve, Swaziland.
Pta Zoo: Leopards and tigers in the Pretoria Zoo, Republic of South Africa.
Jhb Zoo: Lions in the Johannesburg Zoo, Republic of South Africa.
LP: Lions in The Lion Park, Johannesburg, Republic of South Africa.

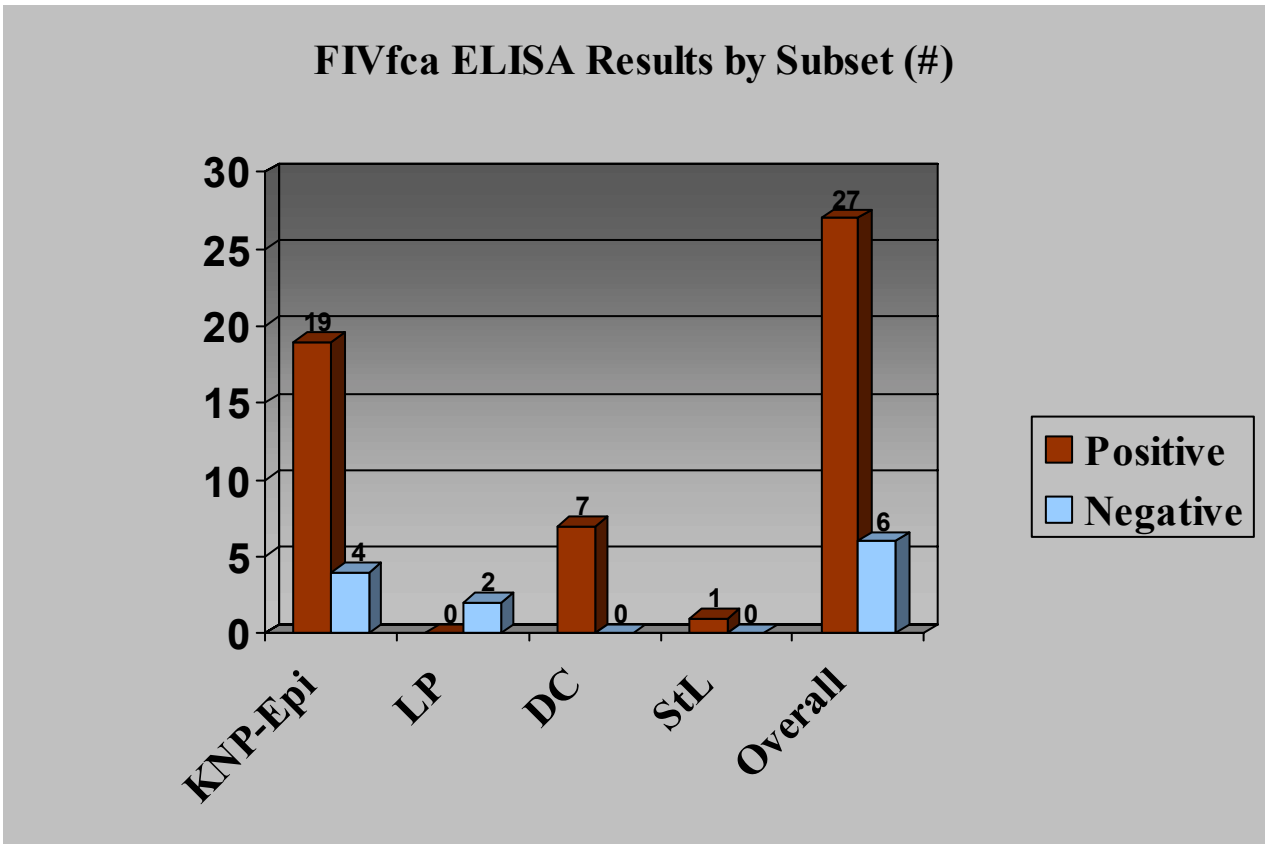


Figure 3.6. FIVfca ELISA results from felid serum samples collected between 2003-2006, according to sample subset (location).

Key:
KNP Epi: Lions in the Kruger National Park, Republic of South Africa who were not tuberculosis tested.
LP: Lions in The Lion Park, Johannesburg, Republic of South Africa.
DC: Domestic cats in Johannesburg, Republic of South Africa.
StL: Lion from the St. Louis Zoo, U.S.A.
Overall: All samples tested.

3.7 Serum Haptoglobins (Hp)

A total of 52 KNP-Epi-S lion serum samples were tested for haptoglobins. The mean concentration was 1600 µg/ml (SD 500 µg/ml; 95% CI: 1465-1743 µg/ml), with a range of 680 µg/ml (KNP-Epi-S FIVple +pos Adult Female) to 3000 µg/ml (KNP-Epi-S FIVple +pos Adult Male). There was no significant difference found between the mean Hp values for FIVple positive (1717 µg/ml) and negative (1536 µg/ml) lions ($t = 0.899$; $P = 0.4, > 0.05$) or between adults (1621 µg/ml) and juveniles (1408 µg/ml) ($t = 1.27$; $P = 0.2, > 0.05$); however a significant difference was found between males (1863 µg/ml) and females (1399 µg/ml) ($t = 3.73$; $P = 0.0, < 0.05$).

3.8 Serum Alpha-one acid glycoproteins (AGP)

A total of 52 KNP-Epi-S lion serum samples were tested for alpha-one acid glycoproteins. The mean concentration was 268 µg/ml (SD 38.8 µg/ml; 95% CI: 257-279 µg/ml), with a range of 260 µg/ml (50 out of 52 samples) to 460 µg/ml (KNP-Epi-S FIVple +pos Male Adult and KNP-Epi-S FIVple –neg Male Juvenile). There were no significant differences found between the mean AGP values for FIVple positive (267 µg/ml) and negative (280 µg/ml) lions ($t = 0.737$; $P = 0.5, > 0.05$), adults (265 µg/ml) and juveniles (282 µg/ml) ($t = 1.24$; $P = 0.2, > 0.05$), or between females (260 µg/ml) and males (277 µg/ml) ($t = 1.54$; $P = 0.1, > 0.05$). It should be noted, however, that if the P-value were raised to 0.1, there would be a significant difference, albeit slight, between female and male lion AGP concentration.

3.9 Epidemiology

Epidemiological data including pride name, approximate location in KNP, animal's identification number, sex, age as estimated by tooth morphology, body weight, and body condition score were collected from 77 of the Kruger National Park lions at the time of blood collection. Of these 77 lions for which epidemiological data were collected: 73 whole blood samples were available for PCR testing; 63 serum samples were tested with the FIVpco ELISA; and 23 serum samples were tested with the FIVfca ELISA.

3.9a Host Factors

Age.--Of the 77 lions sampled in KNP, 58 were classified as adults at 3 years of age or older, and 19 lions were juveniles at less than three years of age (Sunquist 2002), for an age ratio of 3:1 adults to juveniles for the KNP-Epi subset sample overall. Lions in north had an age ratio of 1.6:1 adults to juveniles, and lions in the south had an age ratio of 4.7:1 adults to juveniles.

Sex.--There were 27 males sampled and 50 females sampled from the KNP-Epi subset overall, giving a sex ratio of 1.9:1 females to males. The sex ratio for north lions was 4.2:1 females to males, and for the south was 1.3:1 females to males. There were no bachelor male coalitions sampled from the north prides, however 2 out of the 9 prides sampled in the south were male coalitions, which may help to explain the more equivalent sex ratios in the south, as compared to the north lions.

Body Condition Score.--The average body condition score for the KNP-Epi subset was high overall, at 4.65 out of 5 (Std. Dev. 0.565, Range 3.0-5.0). The BCS for lions in the north was slightly higher, at 4.8 (Std. Dev. 0.450, Range 3.5-5.0), than those in the

south, at 4.6 (Std. Dev. 0.605, Range 3.0-5.0). Only 3 of 77 lions received the lowest score recorded of 3 out of 5, and it can be noted that all three of these lions were part of a bachelor male coalition in the south.

Spatial Variables.--Of the 77 lions sampled from the KNP-Epi subset, 26 lions were from the north, and 51 lions were from the south of KNP.

3.9b Calculation of PCR Test Sensitivity and Specificity

In order to calculate the sensitivity of a new diagnostic test, it is most desirable to compare it against a Gold Standard (GS) test, which for most viruses is virus isolation. However, as virus isolation is neither practical nor possible for the purposes of this study, it was necessary to utilize some of the currently available serological diagnostic tests for FIVple. Therefore, indirect estimates of sensitivity and specificity were calculated according to the recommendations of Thrusfield (2007), which suggests that when there is no GS, a bank of standard tests is used to compare against the results of the new test. The FIVfca ELISA and FIVpco ELISA were used together as the bank of tests to compare with our PCR. If samples tested positive to both tests in the bank, the sample was classified as positive. If samples tested negative to both tests in the bank, the sample was classified as negative. All samples with intermediate results (one positive, one negative) were discarded from the calculations. The calculated values were then referred to as the 'relative' sensitivity and specificity. However, only 21 samples were tested with all three tests (FIVfca ELISA, FIVpco ELISA, PCR), giving a small total sample size for calculations. The relative sensitivity was 79% and the relative specificity was 100%. The positive and negative predictive values were

calculated to be 100% and 57%, respectively. The 2x2 table used for calculations of each value is shown in Figure 3.7.

As the total number of samples tested with both the FIVpco ELISA and the PCR was significantly higher (N=67) than the number tested with all three tests mentioned above, an alternative method of calculating sensitivity and specificity was used in addition to the bank of tests method. This additional method for calculating test values was used for calculations of test sensitivity and specificity when there is no GS available, but the sensitivity and specificity of a reference test (FIVpco ELISA) are presumed known (Staquet 1981; Enoe 2000). Although Bayesian inference or maximum likelihood methods are preferred for the estimation of diagnostic test accuracy when a reference test does not exist, there are other available methods for the estimation of diagnostic test accuracy when a reference test exists that is sub-optimal (i.e. no GS) (Enoe 2000; Gart 1966; Staquet 1981). The results of one such method to estimate diagnostic test accuracy when a sub-optimal reference tests exists is shown in Figure 3.8. The calculated sensitivity and specificity were 49% and 51%, respectively, which are significantly lower than the values calculated according to the banked test method. The prevalence was calculated at 54%, which is similar to the first calculated prevalence.

3.9c Prevalence of FIVple among KNP-Epi Lions

The PCR prevalence of FIVple among the KNP-Epi lion sample subset was calculated using two methods described above for calculating the PCR test sensitivity and specificity (see Figures 3.7 and 3.8). According to the banked test method of

FIVpco & FIVfca ELISA panel as GS (Thrusfield 2007)			
PCR	**(+) pos**	**(-) neg**	
(+) pos	**11**	**0**	**11**
(-) neg	**3**	**4**	**7**
	14	**4**	**Total: 18**
Sen : 11/14=79%	**Spec: 4/4=100%**	**PPV: 11/11=100%**	**NPV: 4/7=57%**
Test Prevalence: 11/18=61%			

Figure 3.7. Calculation of indirect estimates of PCR Sensitivity, Specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV), and FIVple Prevalence by comparison with a bank of standard tests.

FIVpco (R*) as GS**			
PCR (N*)	(+) pos	(-) neg	
(+) pos	29 (a*)	4 (b*)	33 (g*)
(-) neg	8 (c*)	26 (d*)	34 (h*)
	37 (e*)	30 (f*)	Total: 67 (n*)
Sen : 49%	Spec: 51%	Prev : 54%	

*R= Reference test; N= New test; a= animals that were FIVpco and PCR test positive; b= animals that were FIVpco test negative and PCR test positive; c= animals that were FIVpco test positive and PCR test negative; d= animals that were FIVpco and PCR test negative; e= animals that were FIVpco test positive; f= animals that were FIVpco test negative; g= animals that were PCR test positive; h= animals that were PCR test neg.
**FIVpco (Reference test) Sen: 85%; Spec: 100%, from van Vuuren (2003).
***Calculations for Figure 8 determination of PCR Sensitivity, Specificity, and FIVple Prevalence, where the true disease state is unknown:

Calculations for Sensitivity, Specificity, & Prevalence when the true disease state is unknown:
$Sen(N) = g(SpecR) - b / n(SpecR - 1) + e$
$Spec(N) = h(SenR) - c / n(SenR) - e$
$Prev = n(SpecR - 1) + e / n(SenR + SpecR - 1)$

Figure 3.8. Calculation of PCR Sensitivity, Specificity, and FIVple Prevalence when there is no true Gold Standard (GS), and the true disease state is unknown (based on Staquet 1981).

Calculations, the test prevalence was 61%, whereas the Staquet method of calculations gave a test prevalence of 54%. For an overall measure of prevalence, a test bank approach was used, which will be referred to as the combined test prevalence (CTP). If a sample tested positive to all tests, or 2 out of 3 tests, it was classified as a positive result. In addition, if a sample tested positive to PCR and was sequence-confirmed as FIVple, it was classified as a positive. If a sample tested negative to all tests it was classified as a negative. All other intermediate results were discarded from the combined test prevalence calculations. The results of these calculations are shown in Figures 3.9 through 3.13. In addition, the CTP was calculated for the various combinations of the host factors of age and sex and the geographic location of the KNP-Epi lions, and the results are provided in Appendix C.

The overall CTP for KNP-Epi was 69%, with a lower prevalence of 41% in the north, and a higher prevalence of 80% in the south. Adult males had the highest prevalence, at 94%, when combining the factors of sex and age. When geographic locale was included, adult males in the north were highest at a prevalence of 100%. The lowest prevalences were found among juveniles, with male juveniles at a prevalence of 29%. When geographic locale was included, female juveniles in the north and male juveniles in the south were lowest, at 20% and 25%, respectively. Figures 3.12 and 3.13 show the prevalence of FIVple by pride in north and south KNP, respectively.

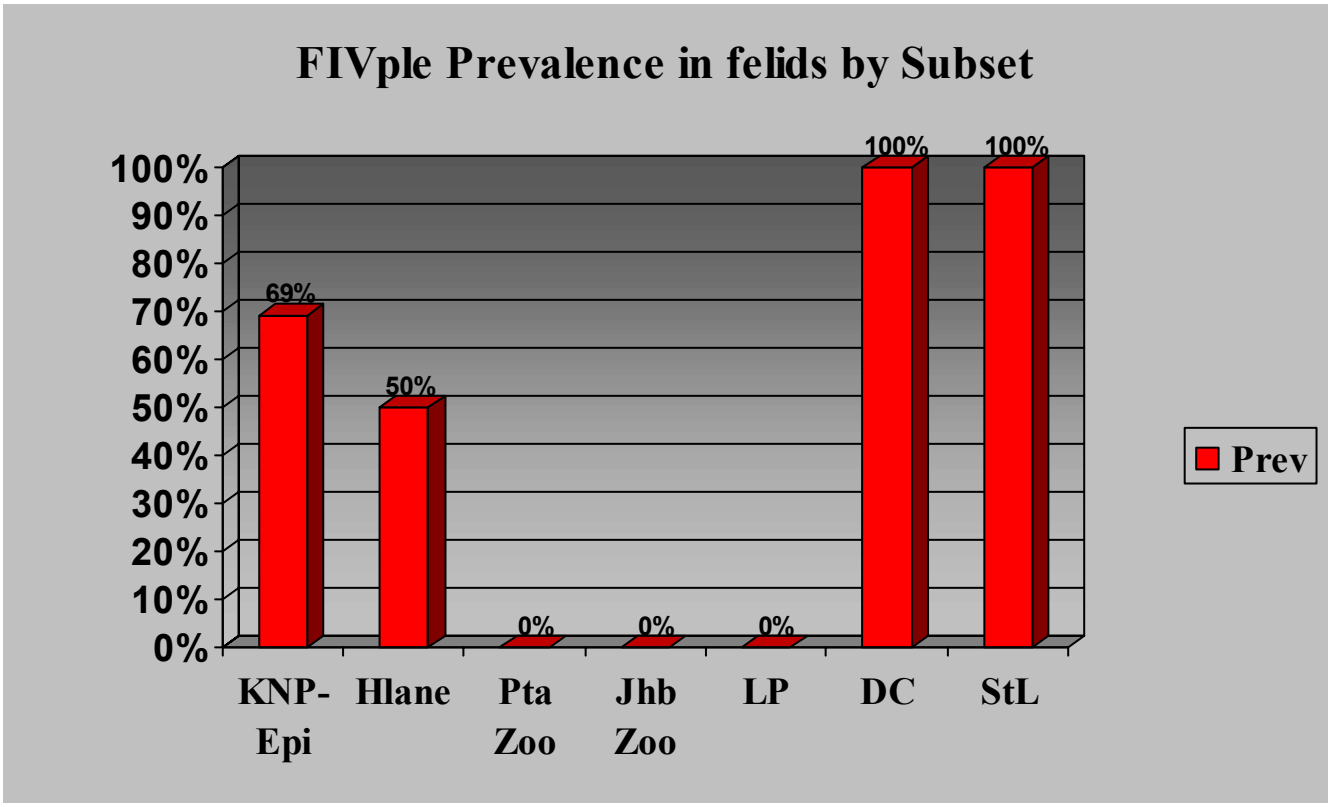


Figure 3.9. Combined Test Prevalence (CTP) according to sample subset (location) of free-ranging and captive wild felids tested between 2003-2006. All sample subsets for which CTP could be calculated have been included.

Key:
KNP Epi: Lions in the Kruger National Park, Republic of South Africa; N= 61.
Hlane: Lions from Hlane Game Reserve, Swaziland; N=2.
Pta Zoo: Leopards and tigers in the Pretoria Zoo, Republic of South Africa; N=7.
Jhb Zoo: Lions in the Johannesburg Zoo, Republic of South Africa; N=3.
LP: Lions in The Lion Park, Johannesburg, Republic of South Africa; N=7.
DC: Domestic cats in Johannesburg, Republic of South Africa; N=7.
StL Lion: Lion at the St. Louis Zoo, U.S.A.; N=1.

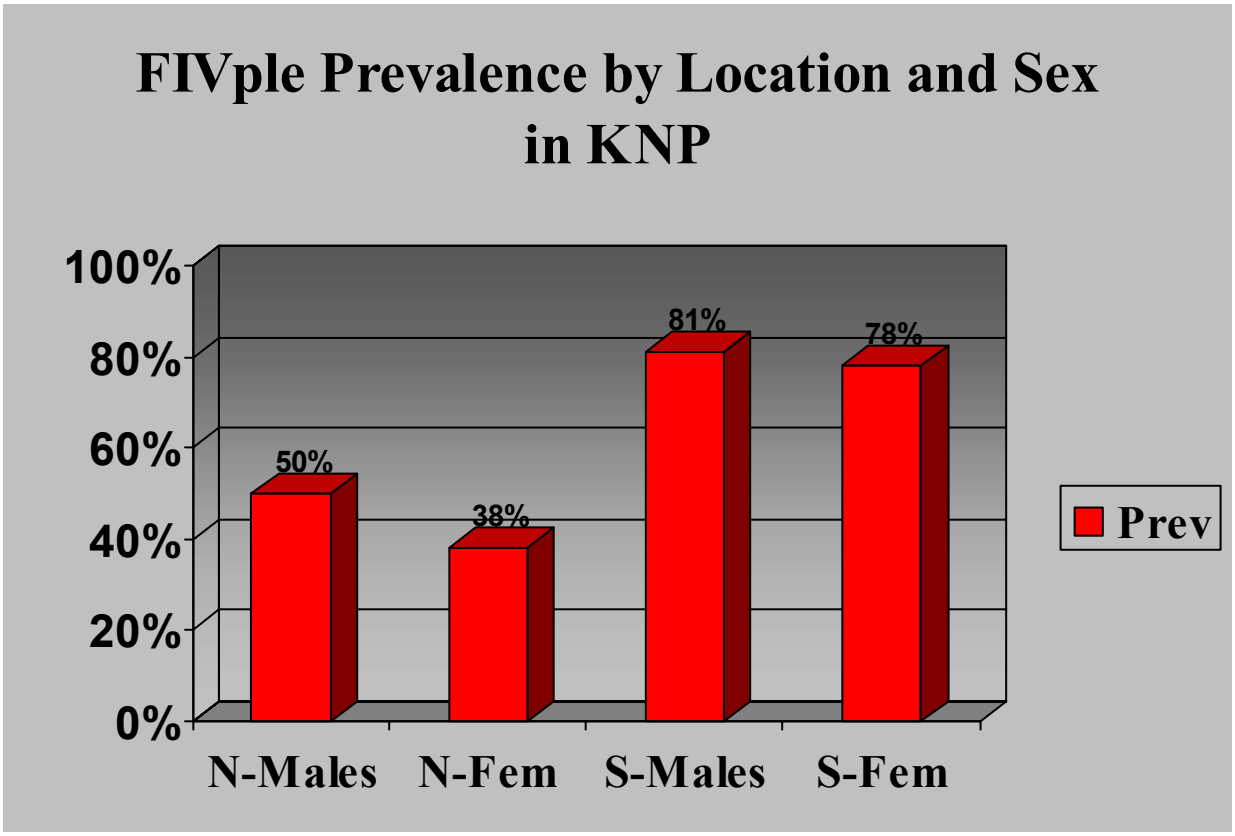


Figure 3.10. Combined Test Prevalence by location and sex of free-ranging lions in Kruger National Park tested between 2003-2006.

Key:
N-Males: Males in KNP-Epi North subset; N= 4.
N-Females: Females in KNP-Epi North subset; N= 13.
S-Males: Males in KNP-Epi South subset; N= 21.
S-Females: Females in KNP-Epi South subset; N= 23.

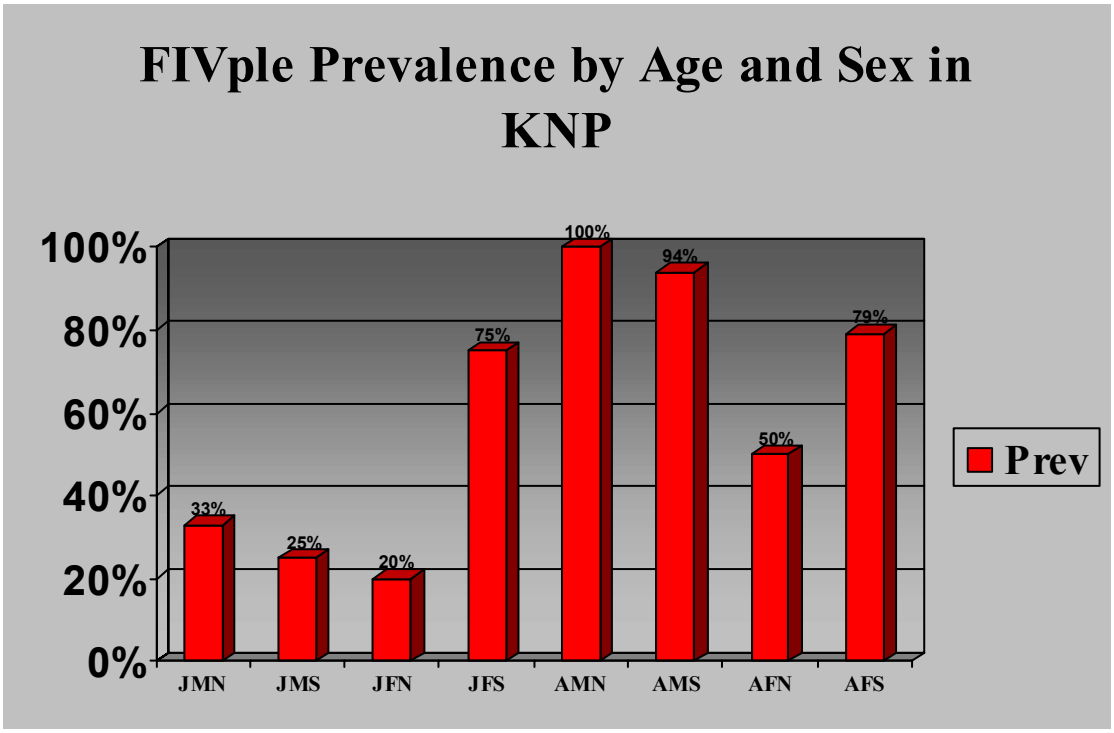


Figure 3.11. Combined Test Prevalence by age and sex of free-ranging lions in Kruger National Park tested between 2003-2006.

Key:
JMN: Juvenile Males in the KNP-Epi North subset; N= 3.
JMS: Juvenile Males in the KNP-Epi South subset; N= 4
JFN: Juvenile Females in the KNP-Epi North subset; N= 5
JFS: Juvenile Females in the KNP-Epi South subset; N= 4.
AMN: Adult Males in the KNP-Epi North subset; N= 1.
AMS: Adult Males in the KNP-Epi South subset; N= 17.
AFN: Adult Females in the KNP-Epi North subset; N= 8.
AFS: Adult Females in the KNP-Epi South subset; N= 19.

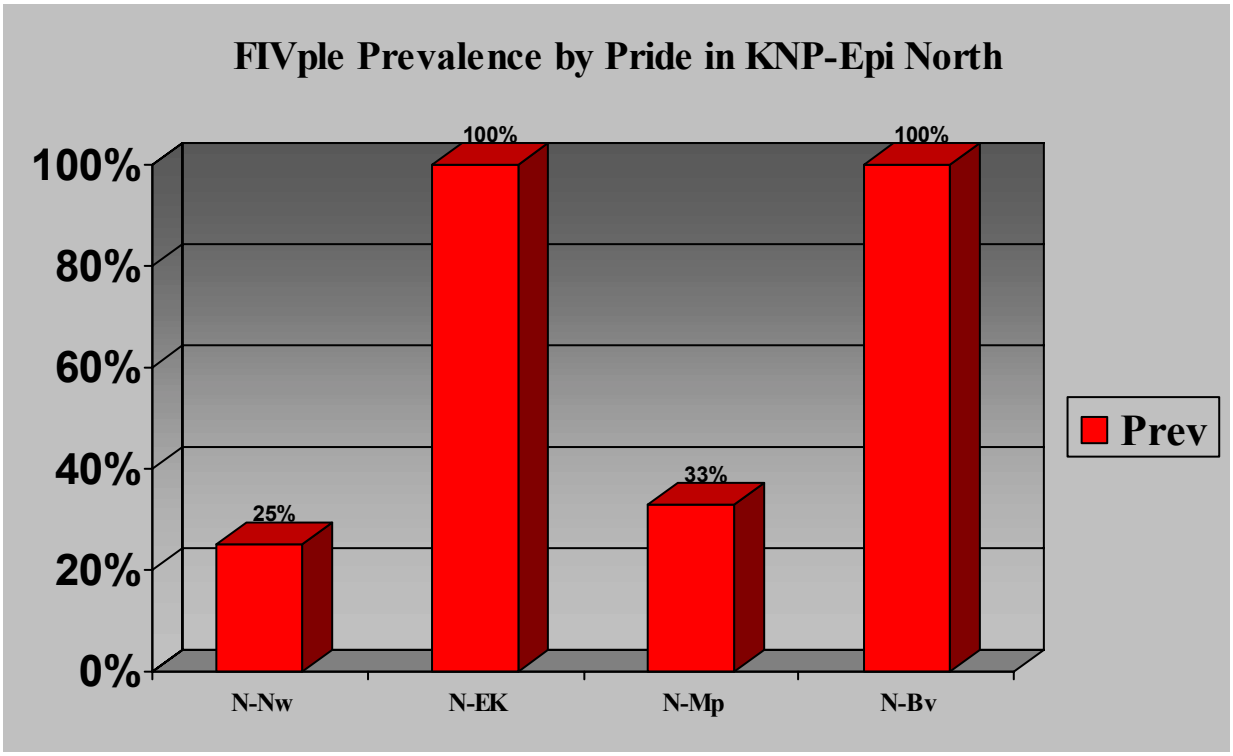


Figure 3.12. Combined Test Prevalence by pride of free-ranging lions in Kruger National Park tested between 2003-2006.

Key:
N-Nw: Nwashitumbe pride; N= 11.
N-EK: Elands Kiel pride; N= 1.
N-Mp: Mpenza pride; N= 3.
N-Bv: Biesiesvlei pride; N= 2.

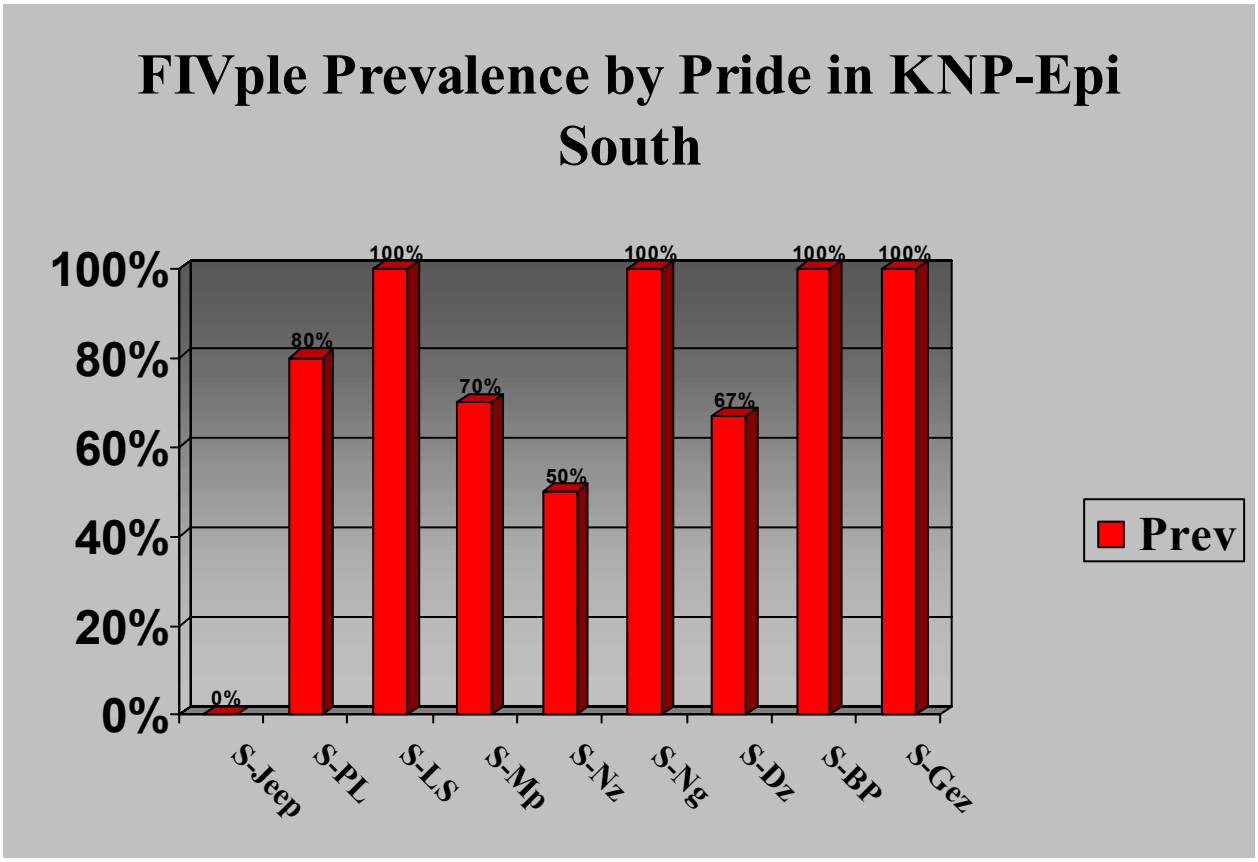


Figure 3.13. Combined Test Prevalence by pride of free-ranging lions in south Kruger National Park tested between 2003-2006.

Key:
S-Jeep: Jeep pride; N= 2.
S-PL: Power Lines pride; N= 5.
S-LS: Lower Sabie pride; N= 7.
S-Mp: Mpanamana pride; N= 10.
S-Nz: Nlanganzwane pride; N= 2.
S-Ng: Ngomondwane pride; N= 6.
S-Dz: Dzuweni pride; N= 6.
S-BP: Bob Picket pride; N= 1.
S-Gez: Gezentombi pride; N= 5.

3.9d Odds Ratios

Odds ratios were calculated to explore the association of prevalence to the host factors of age and sex, and the environmental factor of geographic location of the KNP-Epi lions (Appendix D). Free-ranging lions were 21.50 times more likely to be test positive for FIVple than captive lions. Adults were 5.58 times more likely to be test positive for FIVple than juveniles, with adult males being 35.00 times more likely to be test positive for FIVple over juvenile males. This likelihood increased further, to an OR of 48.00, when comparing adult and juvenile males in the south. Although there was virtually no difference in likelihood of infection for males versus females (OR: 1.48), adult males were 5.89 times more likely to be test positive for FIVple than adult females. There was no difference (OR: 0) in the likelihood of infection between: adult and juvenile males in the north; adult males and females in the north; and between adult males in the north versus the south.

Part Four

DISCUSSION

4.1 Overview and Project Significance to Lion Conservation

The African lion is listed as a vulnerable species by the World Conservation Union (IUCN), whose numbers have declined from an estimated 75,000 to around 30,000, in the last 20 years. Although the species exists throughout sub-Saharan Africa today, it is not a contiguous population, but rather exists as a series of smaller, fragmented subpopulations. Some of these subpopulations are considered to be regionally endangered such as the lions of west Africa, which number only 1,500. These subpopulations of lions have evolved in genetic isolation from the rest of their species, thus there is a limited exchange of genetic material between isolated populations. Genetic exchange is only possible with human intervention such as translocation or the creation of habitat corridors.

The conservation management of free-ranging lions must consider the infectious agents to which they are susceptible. Of these, the lentiviruses are a major focus of investigation. The genetic make-up of the host, as well as the genomic variation and characteristics of the feline lentiviruses are key factors in the dynamic interplay between the host, the agent, and their environment. These three factors—host, agent, and environment, create a triad for which a change in any one factor will have an effect on the others in turn. Thus all three factors should be considered when discussing any health or conservation management strategy for the African lion.

Ecosystem and habitat change has the potential to impact both the lions as hosts as well as the lentiviruses that infect them. For example, as lion habitat is encroached upon, lion density in a particular habitat increases in turn. This may lead to an increase in the frequency of direct encounters with other lions, allowing for increased

opportunities for virus transmission, or for the transmission of other infectious agents (such as bovine tuberculosis among KNP lions) that may in turn impact the immune competence of the lion. Increased lion density also impacts a lion's access to important resources such as food and mates, thus the likelihood of aggression between lions is higher. Individual stress response may also be heightened as a result, and this can have an effect on the immune competence of an individual.

Conversely, if habitat is expanded it can also impact both the host and agent, as resources such as food and access to mates become more scarce. In addition, as habitat is increased to allow for larger territory size, lions are given the opportunity to come into contact with previously segregated populations, allowing for the exchange of genetic material for both host and agent. Humans influence free-roaming lion social systems in a variety of ways, including: 1) human intervention for veterinary or conservation management purposes, such as for lion translocation; 2) the creation of artificial boundaries that dictate where lions can freely roam; 3) illegal lion trophy hunting or poaching along park borders; 4) human encroachment up to park borders, with the introduction of domestic cats along fencelines; 5) and tourism pressures, including the desire to see free-roaming lions at close range, which may in turn influence lion behavior or increase stress among lions.

The lion population of the Republic of South Africa is estimated to be approximately 3,000 lions, with the largest free-ranging population of 2,000 lions in Kruger National Park. The KNP is a fenced habitat with human encroachment to the park's borders, thus limiting the territory size for the population of lions that exist within the boundaries of KNP. However, a new initiative to create a larger transfrontier

conservation area is underway, joining KNP with national parks in the neighboring countries of Mozambique and Zimbabwe. The newly established transfrontier park will be known as the Greater Limpopo Transfrontier Conservation Area (GLTCA), and will allow for the movement of wildlife into new and larger territory. This will create opportunities for lions that have evolved in genetic isolation to come into direct contact with one another, facilitating genetic exchange for both lions as well as for the lentiviruses they harbor. The corridor connecting the KNP as a transfrontier park lies in the north of KNP, thus the impact may initially be observed among the lions of north KNP. As new territory opens for lions in the north, lions in the south of KNP may in turn migrate northward, thus impacting the overall social dynamics, and subsequently the viral epidemiology of FIVple subtypes presently circulating in the entire GLTCA.

This investigation examined lentivirus infection in southern African felids and represents the first epidemiological study of FIVple among the lions of the Kruger National Park, RSA. It is also the first study of this scope that compares genetic material to antibody based methods of FIVple testing on lions in RSA.

4.2 Sample Collection and Sampling Strategy

In this investigation, a total of 107 lions, seven other non-domestic felines, and seven domestic cats were sampled for FIV testing. Out of the 121 animals for which samples were collected for testing, fresh samples were available from only 6 of these animals (five lions from The Lion Park and the St. Louis Zoo lion). For these 6 samples, testing was done within 1-2 days after collection. The remaining 115 animals were sampled in 2003 and 2004. The FIVpco ELISA was performed on the fresh serum

samples and the whole blood and serum samples were then placed in cold storage until testing in this investigation (2006). Although there was no indication that the samples were mishandled or improperly stored, storage time, temperature, as well as repeated freeze/thaw cycles could affect virus titer (Gessoni, 2004). The optimal testing strategy would utilize the freshest whole blood and serum samples available for serological and genetic material testing, however our study indicates that FIVple was detected by PCR after prolonged storage time.

For the KNP-Tb, Hlane, Jhb Zoo, Pta Zoo, and DC subsets (n=36), the original samples were unavailable. The whole blood had been used for a previous study that attempted virus isolation and only the cell pellets in supernatant were available for PCR testing in this investigation. Although cytopathic effects (CPE) were noted among these cultures, Mg^{2+} - dependent reverse transcriptase activity was not monitored. No definitive positives could be concluded from the virus isolation study, as Mg^{2+} - dependent reverse transcriptase activity is required to monitor virus growth in culture (Giannecchini 1996). DNA extractions and PCR testing were performed on the stored cell culture samples, with only a few positive to PCR (five domestic cats, one tiger, and one Hlane lion), although many were positive to the FIVpco ELISA. Because it is unclear if virus was isolated in the samples, no conclusions about the negative PCR results can be made. If present, the amount of provirus in these samples may have been suboptimal.

It is possible that previously published reports of seronegative populations of lions, as well as the seronegative lions identified in our current investigation may be infected with an unidentified subtype of FIVple. Lions in the Serengeti were reported to

have multiple subtypes circulating among their population, and some individuals were infected with multiple subtypes (Troyer 2004). The lions of KNP may also be infected with multiple subtypes of FIVple, some of which could not be detected with the PCR primers used in this study. The successful primers used in this study were not developed based on sequences obtained from any southern African lions. Thus they may not be optimal for detecting subtypes of FIVple unique to southern African lions, but rather were successful in detecting subtypes shared among lions in East Africa, as well as in detecting sequences that may be more conserved among feline lentiviruses.

Sampling of animals was not conducted with respect to a random strategy but was conducted on an opportunistic basis as part of routine surveillance. Thus it may not be a true representation of the population of lions in general, nor of FIVple specifically, from their respective geographical regions. Ideally, sampling strategy would be randomized. However when dealing with a free-ranging wildlife species whose population status is vulnerable, and anesthetic immobilization is required in order to obtain samples, it is necessary and acceptable protocol to sample on the basis of opportunity (Jessup, 2003; Wobeser 1994).

4.3 Importance of Diagnostic Testing

The feline lentiviruses have a high mutation rate and undergo continual evolution within a host and among a population in order to escape host immune defense mechanisms. This high mutation rate results in a variable population of virus subtypes that may in turn acquire new and distinct characteristics of virulence or pathogenicity. If lions are introduced to viral subtypes for which their immune systems have not co-

adapted, an outbreak of disease may ensue. In addition to lentiviral infections in lions, other infectious agents may play a role in shaping the overall immune competence of an individual, and among a population, that may in turn impact the response to infection with FIVple.

Polymerase Chain Reaction as a surveillance tool.—Polymerase chain reaction was used in this investigation to detect lentivirus in biologic samples. PCR allows the researcher or diagnostician to obtain nucleotide sequence information in order to confirm the type of lentivirus, as well as the subtype of FIVple if present. Nucleotide sequencing lends itself to the study of phylogenetics, in order to compare the subtypes present among an individual or pride of lions, or to phylogeography, comparing groups of viruses from animals across regions of the globe. Sequence information may also be used to develop additional primers for PCR that are more specific to each subtype of FIVple. This may help to increase the overall sensitivity for detecting the virus in a population by increasing the chance of detecting the virus in an individual, regardless of subtype.

The disadvantage to using PCR as the sole means of detection of lentivirus infection is due in large part to the inherent heterogeneity of FIV. Ideally, assays using primers specific to all known subtypes or isolates would be developed. It has been demonstrated, both in this research study as well as in the published literature, that the development of a universal PCR to detect a species-specific FIV, whether in domestic cats, lions, or other non-domestic felines, is highly improbable (Crawford 2005). This highlights the advantage of using degenerate primers in order to detect a wider variety of isolates. However this approach may lead to some false negatives, decreasing test sensitivity.

The PCR assay used in this investigation, utilized primers designed from published sequence information of the Pol-RT region of FIVfca, FIVpco, and FIVoma, and was successful in amplifying FIVple and FIVfca in the samples from southern African lions. The identity of the DNA products was confirmed by sequencing and comparison to other published FIV sequences. Additional primer combinations designed from published sequence information of the LTR, gag, and Pol-RNAse regions of FIVfca, FIVpco, FIVoma, and FIVple were not successful in amplifying viral DNA from our samples. This may be due to a variety of reasons. As stated above, there is a high amount of genetic variation among isolates. In addition, degenerate primers were used for PCR, which may lead to decreased amplification efficiency in samples, or to primer annealing during PCR (Linhart, 2002; Rose 1998). Also, the proviral load in some of the samples may have been too low to amplify product or samples may have degenerated from repeated freeze/thaws, mishandling during collection and transport, or during prolonged storage, decreasing the overall amplification efficiency. Although there was sufficient template for amplification of at least some samples using the Pol-RT primers, these latter variables may have contributed to the lack of amplification if primer annealing was suboptimal with the other primer sets. The unsuccessful primer sets targeted different regions of the genome that may have been more variable in their nucleotide sequences. It is possible that other primer sets may have required a different protocol with higher or lower temperatures for annealing, and/or a higher amount of template for successful amplification of FIVple in samples.

Polymerase Chain Reaction to Monitor Cross-Species FIV Transmission.—PCR is an important tool for monitoring the types and subtypes present, as well as for

monitoring the continual evolution of FIVple. Because individual hosts can become co-infected or superinfected with multiple FIV types and/or subtypes, the possibility exists for a new subtype to emerge that may result in a clinical manifestation of disease. The discovery of FIVfca in a captive tiger indicates the possibility of feral cats introducing FIVfca into a captive or free-ranging population of lions, such as the KNP and illustrates the need for the monitoring of subtypes present in a population of lions.

Antibody testing as a surveillance tool.—The FIVpco ELISA, or puma lentivirus ELISA, as it is also known, was reported to have a sensitivity and specificity of 85% and 100%, respectively (van Vuuren 2003). These estimates were calculated based on testing free-ranging lions in RSA, thus it is reasonable to assume that the estimates of sensitivity and specificity will hold true for the purposes of our research. This test uses a synthetic FIVpco peptide (CPFKDICQL, AA 610-618, GenBank U03983) corresponding to an epitope, a highly conserved peptide P237 of FIVfca, of the envelope (Env) glycoprotein. The FIVfca ELISA, or IDEXX Snap Test, has a reported sensitivity and specificity of 100% and 99.5%, respectively, when tested on domestic cats (http://www.idexx.com/animalhealth/testkits/fivfelv/060148205.pdf). There are no known published studies of the sensitivity and specificity of the FIVfca ELISA as tested on African lions, thus we must interpret the FIVfca ELISA test results of our lion samples with caution. The FIVfca ELISA uses recombinant gag proteins that are believed to be highly immunogenic, yet conserved among feline lentiviruses (Coffin 1986; Dowbenko 1985). Overall there was 83% agreement (19/23) between the two forms of antibody testing, with only four discrepant results (see Appendix E). All four of the discrepant results were positive using the FIVfca ELISA, and negative using the FIVpco ELISA. In

addition, among these four discrepant results, two were also positive by PCR, one was negative by PCR, and one was not tested with PCR, as no whole blood was available for testing. Of the two PCR positives, one was sequenced as FIVple. This indicates that the FIVfca ELISA positive is true, and the FIVpco ELISA negative was false. Given these results, it is reasonable to assume that the reported FIVfca ELISA sensitivity of 100% (in domestic cats) may be extrapolated to a higher sensitivity than that of the FIVpco ELISA when testing lion serum samples, with respect to the FIVple isolates circulating among the lions sampled for this investigation. This also indicates that the PCR is likely to be more sensitive than the FIVpco ELISA in detecting FIVple among the lions sampled in this investigation.

The development of an antibody response can take several months to a year to develop after infection, thus antibody detection alone may miss some positives in the early stages of infection (Barr 1996). It is common for wildlife veterinarians and wildlife managers in southern Africa to request feline lentivirus testing before translocating lions, or during routine immobilizations. The University of Pretoria Faculty of Veterinary Science currently uses the FIVpco ELISA alone for diagnosis of FIVple in African lions. Using PCR as an additional diagnostic test for FIV in lions will increase screening sensitivity, and may help to identify which types and/or subtypes are circulating among a population of lions. In addition, it will be useful when translocations are necessary, in order to monitor for changes in the viral epidemiology of a region over time.

4.4 Sequencing and Phylogeny

Thirty-four sequences were obtained out of 52 PCR-positive samples from our study, while 18 amplification products did not produce sequence data, due to problems with low signal strength. A low signal strength results in weak or misshapen signal peaks, and an overall low quality score count for sequences. The design of degenerate primers necessitates a lower melting temperature than what is typically used with non-degenerate primers (Rychlik 1995). A melting temperature that is too low as a result of using degenerate primers can lead to low signal strength during sequencing reactions (Preparata 2004). In addition, homopolymer regions in the sequence of the primer (homopolymer regions were present in the primers used for sequencing) and the target are known to cause drops in signal intensity, noisy data, and/or enzyme slippage, all of which can result in a failed sequence reaction (Hawes 2006). Finally, degenerate primers in general are considered to be problematic when sequencing, as they either do not work, or give poor results (Linhart 2002).

An overall pattern of phylogeography was established when looking at the FIVple sequences phylogenetically. Clades were formed in general based on geographic location in KNP (north versus south), with a common ancestral node, and were well-supported by a high bootstrap value. There was some mixing of sequences between the north and south, indicating the contiguous nature of the KNP ecosystem, allowing for the exchange of virus among the entirety of the KNP lion population. Although there was some sequence clustering by pride among clades, this was not a strict observance, as some clades were composed of sequences from multiple prides of lions. This is plausible,

given the fluid nature of lion social dynamics in KNP, where pride territories may overlap, and males roam freely from pride to pride.

Kruger National Park lion FIVple sequences were compared to those from previously characterized FIVple subtypes A-F. The isolates from lions in the north formed a clade with subtype E (Botswana isolates), while the isolates from lions in the south formed a clade with subtype D (RSA isolates). Subtype A (East and southern African isolates) formed a clade with a mix of both south and north KNP isolates. A clade composed of subtypes B, C, and F (sequenced from lions in East Africa) was formed separate from all KNP isolates. It is possible that the subtypes shared among lions from more than one geographic locale, such as between east and southern Africa, either: 1) infected an ancestor of the modern African lion, and thus the subtypes share a deep ancestry as well; 2) infected African lions before more recent habitat fragmentation restricted lions to the geographic isolation seen today; 3) or infected lions in a new region as a result of translocation of infected lions, as part of repopulation efforts.

Several south KNP sequences formed a clade with a deep ancestral node separate from all other sequences, including all subtypes A-F. This may suggest the possibility of a subtype unique to the population of KNP lions, and more specifically to the lions of south KNP, that has not been previously identified. Alternately, it may indicate the presence of FIVple quasispecies in an individual lion host that differs substantially from other published amplified FIVple Pol sequences. It is interesting to note that the captive-born St. Louis Zoo lion's FIVple sequence formed a clade with these sequences, which may indicate a genetic ancestry to the virus from lions of southern KNP. With these phylogenetic results, it is plausible that some or all of our

seropositive/PCR-negative FIVple results may be attributed to a variety of subtypes and/or isolates present in KNP, not all of which were detected with the primers used in this study.

All domestic cat FIVfca sequences formed a clade well supported by a high bootstrap value, along with a sequence amplified from a tiger at the Pretoria Zoo, identified as an FIVfca isolate. This tiger's lentivirus infection is most likely due to a domestic cat transmission of FIVfca, such as a feral cat coming into direct contact with the captive felines of the Pretoria Zoo.

4.5 Acute Phase Proteins

There are published studies of APP values in domestic felines, however to date there are no known published studies on Hp or AGP values in African lions, whether captive or free-ranging. The APP testing in this investigation followed a protocol standardized for domestic cats, using standard solutions for high and low values based on testing in domestic cats (Cardiotech Services, Inc, Louisville, KY; Giordano 2004). It is not known if the APP measurements from lions in this investigation represent an exact or a relative extrapolation; however, the values can be compared to one another. The results of our APP testing may establish baseline data, and may be used to compare against future studies of acute phase protein values in lions.

There were no significant differences found between the Hp and AGP values of FIVple positive versus negative lions or between adults versus juveniles, in contrast to studies in domestic cats (Duthie 1997; Giordano 2004). Our study revealed a significant difference in Hp values ($P<0.05$) and AGP values ($P<0.1$) between males and females, a

finding not as yet reported in domestic cats. The average Hp value for KNP-Epi-S lions was 1600 +/- 500 µg/ml, compared to the average value of 1300 +/- 640 µg/ml among healthy domestic cats (Giordano 2004). The average AGP value for KNP-Epi-N lions was 268 +/- 39 µg/ml, as compared to the average in domestic cats of 1200 +/- 620 µg/ml. However, when compared to each other, only two out of 52 lion serum samples tested had AGP values that differed significantly from the others, and both of these samples were from male lions. Given the relative low average AGP value overall, as well as the lack of deviation from the mean, it is possible that the AGP values are erroneous, possibly due to a problem with the test kit, or human error. It should be noted that these tests were designed to be performed on domestic felines, thus it is possible that they are not suitable for testing on another feline species such as lions.

For the purposes of this study, no significant difference was found with respect to APP values relative to FIVple status among the lions tested, thus it is not currently recommended that APP values be used as a means of predicting FIVple infection status in this population of lions. However, as more information becomes available on the pathogenicity of FIVple in lions, it may be useful to utilize APP values as a means of assessing the host response to infection, as significant differences are reported in the AGP values of domestic cats with FIVfca as compared to their uninfected counterparts (Duthie 1997).

4.6 Epidemiology of FIVple in Kruger National Park

This project represents the first epidemiological study of the FIVple virus among free-ranging lions of the Kruger National Park, RSA. The estimated population of lions

in KNP was 2000, according to the last recorded census in 2002 (M. van Vuuren, personal communication, 2007). Thus, the population of lions sampled for this study represents roughly 4% of the total population of KNP lions. In general, the density of lions is higher in the south of the park. This may help to explain why there were more males and juveniles sampled in the south, as access to females is greater, and allows males increased opportunities to mate with females in estrous. This may also explain why no bachelor male coalitions were sampled in the north, as bachelor males may be driven to roam further south for access to females, as well as for access to prey species. Approximately twice as many animals sampled for the study were lions from the south. This is partly due to the increased lion density in the south described above, as well as due to the location of the veterinary staff headquarters in the south of KNP, facilitating easier access to the lions of the south than in the more remote north of KNP. The body condition score for lions in KNP was high overall, at 4.6 out of 5. The score was slightly higher for north lions, which may be due to the lower density of lions, with decreased competition for resources among prides and roaming males. The three lions with the lowest BCS were part of the same adult male coalition in the south. All three of these lions were also FIVple positive, so it is plausible to consider that the stresses of life as a bachelor male lion may impact the individual's overall health, which may be reflected in the lower BCS. It may also be noted that muscle wasting was the primary clinical sign noted among FIVple positive lions at the North Carolina Zoo (Bull, 2003).

Combined Test Prevalence.—Roughly two-thirds of the lions sampled from KNP were positive for FIVple by serology and/or virus detection. The prevalence within KNP was higher for lions sampled in the south. This may be due to the increased lion density

in the south, allowing for increased opportunities for virus transmission. Adult males had an extremely high prevalence of FIVple infection as one would expect considering the primary route of transmission is via bite wounds. Overall, juveniles had the lowest prevalence of infection. These results suggest that FIVple infection among the lions studied is most likely primarily due to bite transmission. This may be a more efficient means of transmission than via maternal transmission or other direct contact such as grooming between pride members (saliva), which are established as means of transmission in domestic cats (Burkhard 2002; Burkhard 1997; Dow 1990; Jordan 1995; O'Neil 1995; Pedersen 1993).

Odds Ratios.—Many of the calculated odds ratios were found to be non-significant, however this finding could possibly be due to the small sample sizes in several of the strata. The results of our study showed that free-ranging lions were 21.50 times more likely to be positive for FIVple than captive lions. The captive animals in this study may have been born in captivity from a FIVple negative dam. It may also be possible that the cubs were removed from the dam before transmission occurred, and were raised in isolation from any FIVple positive animals. The opportunities for transmission may be greater among free-ranging populations as compared to lions in captivity. However it is possible for transmission to occur between co-habitating captive lions, or for cross-species transmission to occur from a free-roaming domestic cat to a captive lion.

Among the lions of KNP, adults, and specifically adult males, were more likely to be infected than juveniles (and specifically juvenile males). This likelihood increased further when comparing adult and juvenile males in the south. This agrees with the

above discussion that male lions are more at risk for infection due to the increased propensity for aggressive, roaming behavior. The density of lions is known to be higher in the south of KNP. Thus there may also be an increase in males fighting, and a resultant higher individual stress, as well as increased opportunities for direct contact with other infected lions, as demonstrated in east African lion populations (Heinsohn 1997).

4.7 Conclusions

Due to the dynamic, ever-changing nature of the agent-host-environment relationship, and the inability to gather comprehensive data on free-ranging wildlife, it is difficult to predict whether or not an outbreak of disease with FIVple is possible, or if and when it may occur in a free-ranging population. This alone may be reason enough to demonstrate why monitoring FIVple among populations of lions is crucial, in order to better elucidate and document the patterns of virus-host co-evolution and adaptation over time.

We are only now beginning to understand the complex interplay that exists between the lion, its immune competence, and the co-evolution with FIVple that may or may not contribute to the manifestation of disease in some individuals. Unfortunately when dealing with free-ranging populations of wildlife, there is a limited opportunity to conduct long-term, exhaustive, invasive research into the effects of an infectious agent on a population, thus it is crucial to glean as much information as possible from the limited opportunities available for gathering data. Finally, lion conservation management would

benefit from a more comprehensive knowledge of FIVple evolution, adaptation, and cross-species transmission, for use when planning the development of a newly protected area, when planning strategies for the translocation of animals, as well as to plan for potential treatment and intervention strategies in the case of an outbreak of disease or other health crisis among a population.

4.8 Future Studies

Much work remains in order to better characterize the lentiviruses of lions in general, and southern African lions in particular. The isolates sequenced in this investigation were only partial Pol nucleotide sequences, and cannot be used to represent the viral evolutionary relationships as a whole. This can only be done once the entire FIVple genome has been sequenced for phylogenetic comparison. Other regions of the genome could also be sequenced in order to elucidate potential conservative areas ideal for a more universal PCR. The development of a real-time PCR assay would allow for the monitoring of viral subtype(s), viral load, and shedding patterns among individual free-ranging or captive FIVple positive lions, perhaps using non-invasive sampling techniques such as fecal samples.

No conclusions can be drawn from this investigation with respect to the potential virulence or pathogenetic distinctions between viral subtypes, as little is currently known about the definitive consequences of FIVple infections in African lions. Immunologic studies such as flow cytometry or typing of the major histocompatibility complex (MHC, a complex containing highly polymorphic genes responsible for the regulation and genetic control of immune responses) may uncover potential differences in immune expression among lentivirus positive and negative individuals with regard to either

increased resistance or increased susceptibility to infection or pathogenicity. To this end, FIVple infection status could also be compared to the infection status of bovine tuberculosis, a chronic and often fatal disease currently impacting the lions of the Kruger National Park, Republic of South Africa.

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APPENDIX:

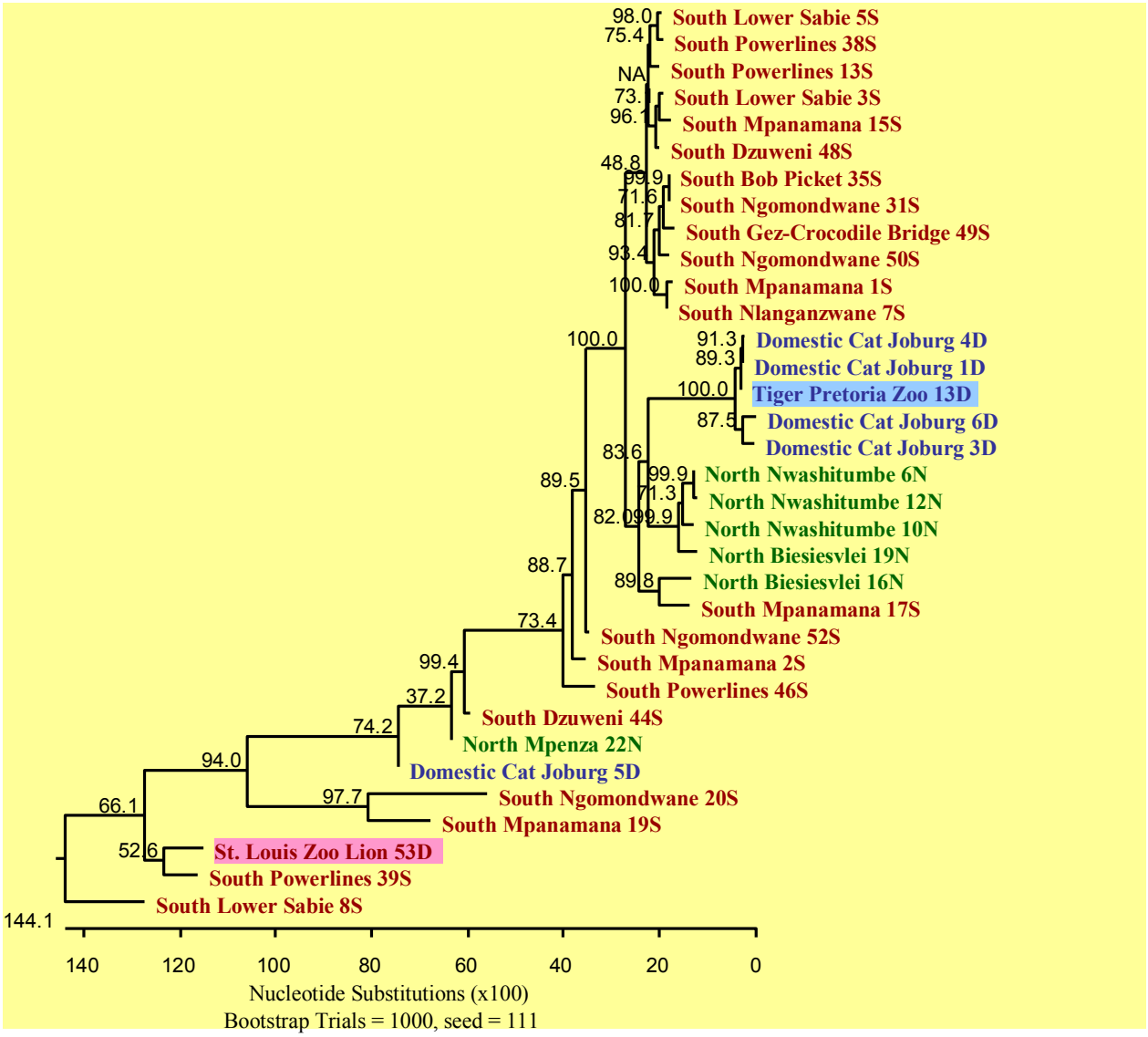
Appendix A. Basic Local Alignment Search Tool results for all nucleotide sequences. Sequence homology and Genbank accession IDs for closest nucleotide sequence also provided.

Sample ID	Virus Identity (BLAST)	Sequence Homology (%)	Genbank Accession for closest NT sequence homology
DC Joburg 1D	FIVfca Australia	96	L16942
DC Joburg 3D	FIVfca Switzerland	95	X57002
DC Joburg 4D	FIVfca San Diego	96	M36968
DC Joburg 5D	FIVfca Australia	96	L16942
DC Joburg 6D	FIVfca Australia	96	L16942
KNP-N Bv 16	FIVple	93	AY878216
KNP-N Bv 19	FIVple	91	AY878208
KNP-N Mp 22	FIVple	89	AY878208
KNP-N Nw 6	FIVple	90	AY878235
KNP-N Nw 10	FIVple	90	AY878208
KNP-N Nw 12	FIVple	90	AY878235
KNP-S BP 35	FIVple	95	AY878224
KNP-S Dz 44	FIVple	91	AY878214
KNP-S Dz 48	FIVple	96	AY878214
KNP-S Gez 49	FIVple	94	AY878224
KNP-S LS 3	FIVple	96	AY878214
KNP-S LS 5	FIVple	96	AY878214
KNP-S LS 8	FIVple	92	AY878214

Appendix A continued: Basic Local Alignment Search Tool results for all nucleotide sequences. Sequence homology and Genbank accession IDs for closest nucleotide sequence also provided.

KNP-S Mp1	**FIVple**	**98**	**AY878224**
KNP-S Mp2	FIVple	98	AY878224
KNP-S Mp 15	FIVple	96	AY878214
KNP-S Mp 17	FIVple	96	AY878216
KNP-S Mp 19	FIVple	95	AY878214
KNP-S Ng 20	FIVple	86	AY878224
KNP-S Ng 31	FIVple	95	AY878224
KNP-S Ng 50	FIVple	95	AY878224
KNP-S Ng 52	FIVple	96	AY878214
KNP-S Nz 7	FIVple	99	AY878224
KNP-S PL 13	FIVple	95	AY878214
KNP-S PL38	FIVple	95	AY878214
KNP-S PL 39	FIVple	96	AY878224
KNP-S PL 46	FIVple	92	AY878216
St. Louis Zoo 53	FIVple	92	AY549255
Pta Tiger 13	FIVfca-US	95	M25381

Appendix B. RT-pol phylogenetic tree of all nucleotide sequences amplified, including samples from lions in the Kruger National Park north and south sections and the St. Louis Zoo, a tiger from the Pretoria Zoo, and domestic cats from Johannesburg, Republic of South Africa.



Appendix C. Combined Test Prevalence of FIVple among the lions of the Kruger National Park (Epi subset), by host factors of age and sex, and by location within Kruger National Park (north or south).

Category (within KNP-Epi)	#Pos	#Neg	Prevalence (%)
Overall	42	19	**69**
North (N)	7	10	**41**
South (S)	35	9	**80**
Females (F)	22	13	**63**
Males (M)	15	6	**71**
Adults (> or = 3ya) (A)	31	10	**79**
Juveniles (< 3ya) (J)	5	9	**36**
North-Females	5	8	**38**
North-Males	2	2	**50**
North-Adults	5	4	**56**
North-Juveniles	2	4	**33**
South-Females	18	5	**78**
South-Males	17	4	**81**
South-Adults	31	5	**86**
South-Juveniles	4	4	**50**
Female-Adults	19	8	**70**
Female-Juveniles	4	5	**44**
Male-Adults	14	1	**94**
Male-Juveniles	2	5	**29**
North-Female-Adults	4	4	**50**
North-Female Juveniles	1	4	**20**
South-Female-Adults	15	4	**79**
South-Female-Juveniles	3	1	**75**
North-Male-Adults	1	0	**100**
North-Male-Juveniles	1	2	**33**
South-Male-Adults	16	1	**94**
South-Male-Juveniles	1	3	**25**

Appendix D. Odds ratios for FIVple positivity among free-roaming lions in Kruger National Park, according to host factors and geographic location.

Category (within KNP-Epi unless otherwise noted)	Odds Ratio	OR 95% CI	p-value
Free-Ranging (KNP-Epi) vs. Captive Lions	21.50	2.63-953.99**	0.00033*
South (S) vs. North (N)	5.56	1.42-22.61	0.0040
Male (M) vs. Female (F)	1.48	0.40-5.60	0.52
Adult (A) vs. Juvenile (J)	5.58	1.27-25.78**	0.010*
N-M vs. N-F	1.60	0.09-28.34**	0.99*
S-M vs. S-F	1.18	0.21-7.00**	0.99*
S-F vs. N-F	5.76	1.05-32.55	0.030*
S-A vs. N-A	4.96	0.70-34.21**	0.063*
M-A vs. F-A	5.89	0.64-279.87**	0.12*
F-A vs. F-J	2.97	0.48-18.82**	0.23*
M-S vs. M-N	4.25	0.22-71.26**	0.23*
M-A vs. M-J	35.00	1.87-1766.69**	0.0043*
J-S vs. J-N	2.00	0.15-33.32**	0.63*
J-F vs. J-M	2.00	0.17-30.82**	0.63*
J-M-N vs. J-M-S	1.50	0.01-78.25**	0.99*
J-F-S vs. J-M-S	9.00	0.21-626.24**	0.49*
A-M-S vs. J-M-S	48.00	1.43-2663.96**	0.011*
J-F-S vs. J-F-N	12.00	0.31-782.99**	0.21*
A-F-S vs. J-F-S	1.25	0.02-21.43**	0.99*
J-M-N vs. J-F-N	2.00	0.02-58.70**	0.99*
A-M-N vs. J-M-N	0.00	0.00-39.00**	0.99*
A-F-N vs. J-F-N	4.00	0.21-245.34**	0.56*
A-F-S vs. A-F-N	3.75	0.45-30.87**	0.18*
A-M-S vs. A-F-S	4.27	0.35.222.99**	0.34*
A-M-N vs. A-F-N	0.00	0.00-48.75**	0.99*
A-M-N vs. A-M-S	0.00	0.00-663.00**	0.99*

Odds Ratios, p-value, and 95% confidence limits calculated using Epi Info Statcalc version 3.4.1 (EpiInfo Website; http://www.cdc.gov/epiinfo/about.htm)
*p-values with an (*) indicate the fisher exact two-tailed test; all other p-values are Maentzel-Hansel (EpiInfo Statcalc).
** indicates exact CI used; all other CI are Cornfield's 95% CI (EpiInfo Statcalc).
***values in red indicate an odds ratio of statistical significance.

Appendix E. Results of samples tested with all three tests for feline lentivirus in lions of the Kruger National Park, Republic of South Africa.

Sample ID	FIVfca ELISA (pos/neg)	FIVpco ELISA (pos/neg)	PCR (pos/neg)
21848	Neg	Neg	Neg
A251B* **	Pos	Pos	Neg
D062B**	Pos	Neg	Neg
E3D00	Pos	Pos	Pos
550205	Pos	Pos	Pos
534E04* **	Pos	Pos	Neg
B176607* **	Pos	Pos	Neg
67B35	Pos	Pos	Pos
40085E	Pos	Pos	Pos
33833	Pos	Pos	Pos
F1F6314	Pos	Pos	Pos
D655B	Pos	Pos	Pos
321408	Pos	Pos	Pos
D184C4C	Pos	Pos	Pos
B2C2D	Neg	Neg	Neg
5A 5278	Neg	Neg	Neg
95270	Pos	Pos	Pos
249 A3A	Pos	Pos	Pos
44 A559AF76	Neg	Neg	Neg

Appendix E continued: Results of samples tested with all three tests for feline lentivirus in lions of the Kruger National Park, Republic of South Africa.

C4560*	Pos	Neg	Pos
80263*	Pos	Neg	Pos
E4223****	Pos	Neg	No whole blood available
Bill-LP	Neg	Neg	Neg
Ben-LP	Neg	Neg	Neg

*Discrepancy between FIVfca and FIVpco ELISA; PCR agreement with FIVfca ELISA
**Discrepancy between FIVfca and FIVpco ELISA; PCR agreement with FIVpco ELISA
***Discrepancy between PCR and ELISAs; agreement between FIVfca and FIVpco ELISAs.
****Discrepancy between FIVfca and FIVpco ELISA; no whole blood available for PCR

Vita

Hayley R. Adams was born and raised in Tennessee. She graduated from the University of Tennessee with a B.S. in Zoology in 1997. She entered the University of Tennessee College of Veterinary Medicine in 1997 and graduated with a Doctor of Veterinary Medicine in 2001.

She went on to complete an internship in Small Animal & Emergency Medicine in Mesa, Arizona, followed by a position as an Associate Veterinarian in Small Animal General Practice in East Tennessee. In 2003 she entered into the University of Tennessee College of Veterinary Medicine's Comparative and Experimental Medicine Program, and received a Doctor of Philosophy degree in 2007. Her dissertation was titled, "Feline Lentivirus: Molecular Analysis and Epidemiology in Southern African Lions (*Panthera leo)*". She is currently preparing for board certification from the American College of Veterinary Preventive Medicine and the American College of Veterinary Microbiology.