GENETIC, ANTIGENIC AND PHENOTYPIC COMPARISON OF HERPESVIRUSES
ISOLATED FROM DOMESTIC AND WILD FELIDS

By

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DEDICATION

I can do all things through Christ who strengthens me.

Philippians 4:13

Dedicated with love to my husband Clément Kandu-Lelo and my children

Lydia, Serge and Betty Kandu-Lelo for their sacrifice and support during my long
absence from home while doing the MSc.
DECLARATION

Apart from the assistance received as reported in the acknowledgment section, this dissertation is the original work of the author.

No part of the dissertation has been previously submitted and is not currently being submitted in candidature for any other degree at any university.

...........................................  Date........../ ........ / 2009

Signature of candidate
ACKNOWLEDGEMENTS

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Special thanks to Prof E. Venter my co-supervisor who welcomed me into her laboratory with very little experience and allowed me to accomplish my dream. I have learned so much over the past few years.

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Feline herpesviruses are endemic in free-ranging lions in South Africa. Serological surveillance among free-living felids revealed high levels of exposure to the virus. However, clinical disease in wild felids following FHV-1 infection has been only described in captive populations and reported to be similar to that in the domestic cat. To expand the epidemiological understanding of feline herpesviruses in felids and for disease control, three strains of FHV-1 isolated from a domestic cat (Felis catus) a cheetah (Acinonyx jubatus) and an African wild cat (Felis silvestris) have been compared to determine their relatedness.

A region of the herpesvirus DNA polymerase gene was amplified in a nested PCR with consensus degenerate primers to confirm the identity of the isolates. The genetic relatedness were investigated by comparing patterns of genomic DNA cleaved with restriction enzymes SalI and KpnI and the DNA fingerprints generated by different RAPD primers. For antigenic relationships, a panel of nine monoclonal antibodies prepared against a vaccine strain used against domestic cats were tested in a microneutralization assay. In addition, the phenotypic characteristics of the
isolates were also compared by their ability to produce plaques in CrFK monolayer cell cultures.

With restriction enzyme analysis, it was not possible to make a comparison due to lack of digestion of the genomic DNA of the domestic cat isolate. However, the RAPD-PCR revealed that isolates were closely related but distinct from each other. Only two monoclonal antibodies reacted with the wild isolates; an effect similar to a toxic effect on cell was observed with the domestic isolate. No significant differences of plaque production were observed among the strains.

This study provides evidence of a closer evolutionary relationship between the three isolates. The results of the relationships based on the genetic and phenotypic characterization agreed well and both indicated that the viruses from the domestic and wild felids are different but have a high degree of similarity.
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<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>CC</td>
<td>Cell control</td>
</tr>
<tr>
<td>ChHV</td>
<td>Cheetah herpesvirus</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>CrFK</td>
<td>Crandell Reese feline kidney</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNA poly</td>
<td>DNA polymerase</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetra-acetic acid</td>
</tr>
<tr>
<td>est</td>
<td>Estimate</td>
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<tr>
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<td>Figure</td>
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<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FHV-1</td>
<td>Feline herpesvirus type 1</td>
</tr>
<tr>
<td>HSV-1</td>
<td>Herpes simplex virus type 1</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>gB</td>
<td>Glycoprotein B</td>
</tr>
<tr>
<td>gC</td>
<td>Glycoprotein C</td>
</tr>
<tr>
<td>gD</td>
<td>Glycoprotein D</td>
</tr>
<tr>
<td>gE</td>
<td>Glycoprotein E</td>
</tr>
<tr>
<td>gG</td>
<td>Glycoprotein G</td>
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<tr>
<td>gH</td>
<td>Glycoprotein H</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>gl</td>
<td>Glycoprotein I</td>
</tr>
<tr>
<td>gp</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>LATs</td>
<td>Latency-associated transcripts</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>Mg</td>
<td>Magnesium</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mM</td>
<td>Milli molar</td>
</tr>
<tr>
<td>MS</td>
<td>Molecular screening</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>NaAC</td>
<td>Sodium acetate</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>Ammonium sulphate precipitation</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>ORFs</td>
<td>Open reading frames</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS⁺</td>
<td>Phosphate buffered saline with calcium and magnesium</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming unit</td>
</tr>
<tr>
<td>pmol</td>
<td>Picomole</td>
</tr>
<tr>
<td>RAPD</td>
<td>Random amplified polymorphic DNA</td>
</tr>
<tr>
<td>RE</td>
<td>Restriction enzyme</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RR</td>
<td>Ribonucleotide reductase</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperatures</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SNT</td>
<td>Serum neutralization test</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate EDTA</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Tissue culture infective dose 50</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TK</td>
<td>Tymidine kinase</td>
</tr>
<tr>
<td>TNE</td>
<td>Tris-sodium chloride EDTA</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Tris-hydrochloride</td>
</tr>
<tr>
<td>U&lt;sub&gt;L&lt;/sub&gt;</td>
<td>Unique long</td>
</tr>
<tr>
<td>U&lt;sub&gt;S&lt;/sub&gt;</td>
<td>Unique Short</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VP</td>
<td>Viral protein</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
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CHAPTER 1

GENERAL INTRODUCTION

1. Overview

Feline herpesvirus type 1 (FHV-1) infection is one of the most frequently detected viral infections of domestic cats (Gaskell & Willoughby 1999), and was first described in 1958 by Crandell and Maurer as a new feline virus associated with intracellular inclusion bodies and upper respiratory tract infections. The name feline viral rhinotracheitis (FVR) was proposed for the disease and the domestic cat was perceived to be the only host of the virus (Bistner, Carlon, Shively & Scoll 1971). The first documented case of infection and clinical signs in wild felids with FHV-1 was reported in 1970 in cheetahs in Australia (Sabine & Hyne 1970) and in different felid species in the Cincinnati Zoo/USA, although no definitive isolation of the virus was done in the latter case (Theobald 1970). A second documented isolation of FHV-1 was from a clouded leopard colony at the St Louis zoological park/Missouri in 1977 (Boever, McDonald & Solorzano 1977). Subsequent reports of clinical disease in cheetahs and other wild felid species were limited to captive animals. The confirmation of FRV by detection of FHV-1 in wild felids is not frequent; but an increasing amount of serological data provide evidence that FHV-1 or a closely related herpesvirus is present among wild felids (Spencer & Morkel 1993; Van Vuuren, Stylianides, & Du Rand 1997; Munson, Marker, Dubovi, Spencer, Evermann & O'Brien 2004). From the published literature, it would seem reasonable to come to the conclusion that feline herpesvirus is a threat especially to wild felids in captivity.

Feline herpesvirus type 1 is today accepted as a common pathogenic virus of felid species causing primarily an upper respiratory tract disease in both domestic and wild felids, and has been experimentally associated with genital tract infections and abortion. The latter is attributed to non-specific effects of the severe debilitating
upper respiratory disease and not to the effects of the virus itself (Gaskell, Dawson & Radford 2006). In addition, its role in more chronic ocular disease and skin lesions is increasingly being recognized (Gaskell, Dawson, Radford & Thiry 2007). In general, FVR is less commonly encountered in isolated felids than in colony cats (Costes, Brande, Thiry & Vanderplasschen 2007).

The virus has a very strict host range and infects only members of the family *Felidae* and cells of feline origin. Feline herpesviruses have the ability to establish a life-long infection usually achieved by latency in sensory ganglia after primary infection from which virus may reactivate with or without recrudescence and viral shedding leading to infection in susceptible hosts (Murphy, Gibbs, Horzinek & Studdert 1999; Gaskell et al. 2006). Vaccination provides good protection against clinical disease. However, none of the vaccines so far developed has been able to provide complete protection. They prevent the appearance or reduce the severity of clinical signs after challenge, but neither prevents reinfection with virulent strains nor protect against latent infection (Costes et al. 2007; Gaskell et al. 2007).

Feline viral rhinotracheitis is endemic in free-ranging lions in South Africa (Spencer & Morkel 1993; Van Vuuren, Goosen & Rogers 1999). Clinical disease in wild felids following infection with FHV-1 has so far been described only in captive populations, although serological surveillance among free-living felids revealed high levels of exposure to the virus (Van Vuuren et al. 1997). Another published survey of FHV-1 infections reported a seroprevalence of 91% in lions in the Kruger National Park (Spencer 1991).

No comparative studies on the characteristics of FHV-1 in domestic and wild felids in South Africa have been done and little is known about their epidemiology.

For epidemiological purposes and disease control, it is deemed important to determine if the FHV-1 of domestic and wild felids are identical or distinct.
1.1. Feline herpesvirus type-1

1.1.1. Classification

Feline herpesvirus type 1 is a large, enveloped DNA virus, and a member of the family Herpesviridae, subfamily Alphaherpesvirinae and genus Varicellovirus (Roizman & Pellet 2001).

1.1.2. Structure and morphology

Herpesvirus virions are enveloped, about 150 nm in diameter and consist of four distinguishable substructures (Fig.1.1). The core which contains the genomic double stranded DNA is wrapped as a toroid (Roizman & Knipe 2001) and appears to be suspended by fibrils that are anchored to the inner side of the capsid, the icosahedral capsid assembly of 162 capsomers arranged in a T=16 symmetry and composed of structural proteins; the tegument, a dense layer of proteins surrounded by a typical lipoprotein envelope derived from host cell membranes with short glycoprotein spikes on its surface accessible to antibodies (Murphy et al. 1999; Roizman & Pellet 2001; Jerome & Morrow 2007; Mettenleiter, Keil & Fuchs 2008). All virion proteins are made after infection and no host protein could be detected in purified virion preparations (Roizman & Knipe 2001).

Feline herpesvirus-1 is relatively fragile in the external environment. Because the viral envelope is lipid-rich, the virus is susceptible to most commercially available lipid solvents and is readily inactivated within 3 hours at 37 °C, and exposure to pH < 4. At low temperatures, the virus has shown to remain infective for 154 days at 4 °C,
although its survival is shorter at higher temperatures; 33 days at 25 °C and 4-5 minutes at 56 °C (Jerome & Morrow 2007)

Fig.1.1. Schematic drawing of a herpesvirus virion from Flint, Enquist, Racaniello & Skalka (2004)

1.1.3. Replication in cell culture

During herpesvirus lytic infection, virions attach via glycoprotein B (gB) and gC to heparin-sulphate receptors on the surface of the host cell (Maeda, Yokoyama, Fujita, Maejima & Mikami 1997) followed by the interactions of the gD with one of several cellular receptors (Roizman & Knipe 2001; Spear & Longnecker 2003). The fusion of the viral envelope with the plasma membrane follows rapidly, the de-enveloped capsid is translocated to the nuclear pores and the DNA is released into the nucleus. Tegument proteins VP6 and UL41 prime the cell for synthesis of viral components. The UL41 protein is involved in degradation of mRNAs to affect the virus-induced
host cell shutoff, whereas the VP16 protein acts as a transcriptional activator and stimulates viral gene expression of immediate early (IE or α) genes, which in turn trans-activate the expression of the early (E or β) genes. The late genes (L or γ) are transcribed after viral DNA synthesis has been initiated and encode mainly structural components of the virion. After viral gene expression and DNA synthesis, capsid proteins are translocated from the cytoplasm to the nucleus. Progeny DNA is packaged into preassembled capsids with the aid of DNA binding proteins; the virion matures and acquires infectivity by budding through the inner lamella of the nuclear membrane (Roizman & Knipe 2001; Mettenleiter 2006).

1.2. Characteristics of FHV-1

1.2.1. Genetic characteristics

The FHV-1 genome is a linear, double-stranded DNA molecule of 134 kilobase (Kb) pairs with 72 genes, a number of which encode glycoproteins (Maeda, Horimoto & Mikami 1998). Feline herpesvirus type 1 has a type D genome structure (Fig. 1.2) (Roizman & Pellet 2001) characterized by two covalently joined segments: long and short unique regions (UL and US). The L segment is 104 kb in size and is composed of unique DNA. The adjacent S segment is approximately 30 kb in size and contains a central portion of unique DNA (US) of 8 kb in size bracketed by inverted repeat sequences of 11 kb in size (internal repeat, IRs; terminal repeat, TRs) (Rota, Maes & Ruyechan 1986b; Grail, Harbour & Chia 1991). This results in the formation of two isomers of the genome during replication, which is distinguished by the orientation of the US. The biological relevance of the formation of two genome isomers is unclear (Roizman & Batterson 2007; Mettenleiter et al. 2008).

Analysis of the nucleotide sequence of FHV-1 genome has identified 31 open reading frames (ORFs); 26 have been localized (15 in the UL segment, 6 in the US and 5 in the IRs or TRs) and only 19 characterized (Costes et al. 2007). All ORFs
appear to be transcribed and exhibit a relatively high degree of homology with herpes simplex virus type 1 (HSV-1) considered to be the prototype of the \textit{alphaherpesvirinae} subfamily, whereas the \textit{UL}45, \textit{US}8.5 genes are unique to the FHV-1 (Willemse, Strijdveen, van Schooneveld, Van den Berg & Sondermeijer 1995).

The ORFs \textit{US}4 (gG), \textit{US}6 (gD), \textit{US}7 (gl) and \textit{US}8 (gE) appear to be conserved with minor variations throughout the alphaherpesviruses. Except for the gD gene, none of the genes encoded by the Us region seems to be critical for viral infectivity; they are non-essential (late genes) and are dispensable for growth in cell culture (Spatz, Rota & Maes 1994), although the \textit{US}8.5 does not seem to be critical for virus propagation in cell culture (Willemse \textit{et al.} 1995). The \textit{UL} region encodes genes essential for replication (immediate and early immediate genes), however, the function of many of them remains unknown (Baines & Roizman 1991; Willemse \textit{et al.} 1995).

\textbf{Fig.1.2.} Schematic organization of the genome of FHV-1.

(a) FHV-1 has a type D herpesvirus genome, with a unique long segment (\textit{UL}), internal and terminal repeat sequences (IR and TR), which flank the unique short segment (\textit{US}).

(b) Schematic representation and localization of detected ORFs. Horizontal arrows indicate transcriptional direction (from Gaskell and Willoughby 1999)
Although only little information is currently available on the comparison of FHV-1 strains from different wild felids, studies on genetic comparisons between domestic FHV-1 isolates and C-27, the prototype strain of FHV-1 have been reported. Feline herpesvirus type-1 isolates appear to be relatively similar to one another (Radford, Gaskell & Dawson 2004; Gaskell et al. 2007). Similarly Herrmann, Gaskell, Ehlers & Ludwig (1984) reported uniformity in the restriction enzyme profiles of 12 domestic cat isolates and a vaccine strain of FHV-1.

It was suggested that the herpesvirus of cheetah (ChHV) represents a distinct strain of FHV-1. When restriction endonuclease analysis was performed, the ChHV genome appeared relatively homogenous to C-72 at the genetic level and contained little restriction fragment size variability (Scherba, Hajjar, Perinikoff, Sundberg, Basgall, Leon-Monzon, Nerurkar & Reichmann 1988). In 1999, Pratelli, Tempesta, De Palma, Martella & Buonavoglia could not establish if the disease in a zoo was caused by an FHV-1 strain reactivated in a wild felid or by a strain transmitted by domestic cats living in the zoo. The restriction enzyme analysis did not clarify this aspect but confirmed a general stability of the FHV-1 genome.

However, variations between domestic FHV-1 isolates have been reported in other studies (Grail & Harbour 1990; Grail et al. 1991; Maeda, Kawaguchi, Ono, Tajima, & Mikami 1995a, b). Differences were found between restriction enzyme patterns of some attenuated strains used in vaccines and some apparently more virulent challenge strains (Horimoto, Limcumpao, Xuan, Ono, Maeda, Kawaguchi, Kai, Takahashi & Mikami 1992; Maeda et al. 1995a). These variations occurring in FHV-1 isolates could be associated with mutations observed in genes. Such mutations have been found in the UL17 gene of FHV-1 DNA between isolates from two different geographic regions of Japan (Hamano, Maeda, Kai, Mochizuki, Tohya & Akashi 2005). Moreover, Maeda et al. (1995a) and Hamano, Maeda, Mizukoshi, Mochizuki, Tohya, Akashi & Kai (2004) observed that some FHV-1 strains have a genetic rearrangement within the gene gC and vaccine strains are characterized by the absence of a 36 kDa (kilodalton) immunogenic protein.
Most of the variations between strains of FHV-1 occur in fragments at the viral termini and within the internal inverted repeat sequences (Rota et al. 1986b; Horimoto et al. 1992). The short segment \( U_S \) is relatively stable and \( U_L \) is variable with respect to restriction sites, DNA sequences and in coding for the majority of variable polypeptides (Grail et al. 1991). Mutations have been suggested to occur sporadically and thus may not be readily detected (Grail & Harbour 1990; Horimoto et al. 1992). These genetic variations are not as prominent as in other herpesviruses (e.g. bovine herpesvirus) where differences in the pathogenicity of isolates may be correlated with restriction enzyme patterns (Pauli, Gregersen, Storz & Ludwig 1984).

The apparent low rate of variability among FHV-1 isolates suggests that the genome has evolved at a fairly constant state, perhaps through interaction with host cell DNA during latency, or reflecting some intrinsic property of the virus (Grail et al. 1991).

Restriction sites have been found not to alter in epidemiologically related isolates (Roizman & Tognon 1983). The loss or gain of restriction sites as a result of passage has not yet been reported (Herrmann et al. 1984). The DNA restriction patterns of equine herpesvirus-1 strains did not alter during more than 100 passages of \textit{in vitro} subcultivation in equine cells (Allen, Yeargan & Bryans 1983). It was concluded that varicella zoster virus DNA was sufficiently stable after multiple passages in cell culture (Zweerink, Morton, Stanton & Neff 1981). Furthermore, Herrmann and colleagues (1984) could not find any evidence that the FHV-1 genome alters during a period of latency.

Previously studies on genetic variation in FHV-1 isolates were performed using restriction endonuclease analysis. However random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) was also used in virology to analyze closely related isolates. This technique was successfully used to study the relationship between bovine herpesviruses (Afonso, Ortega, Redondo, Trindade & Barbosa-Stancioli 2007) suggesting that it may provide a useful tool for isolate discrimination within the FHV-1 group. However, no data is available for FHV-1.
1.2.2. Antigenic characteristics

Strains of FHV-1 are antigenically similar and belong to one serotype (Povey 1979; Scherba et al. 1988). The glycoproteins of FHV-1 are structural components of the virion envelope and are expressed on the surface of infected cells. They are important antigenic determinants of the subtype specificity of the virus. Antigenic strain differentiation is often related to variation in these glycoproteins of the virus envelope (Horimoto, Limcumpao, Tohya, Takahashi & Mikami 1990; Spatz et al. 1994). Feline herpesvirus type 1 glycoproteins are involved in important functions such as attachment and infection of susceptible cells and cell-to-cell spread. They also play an important role in the induction of virus neutralizing antibodies and cell-mediated immunity. Because of their locations in the viral envelope, and on the surfaces of infected cells, glycoproteins are major targets of the host immune responses (Roizman & Knipe 2001; Jerome & Morrow 2007).

Feline herpesvirus type 1 virions contain 23 viral proteins; seven are associated glycoproteins designated gB, gC, gH located in the UL region and gD, gE, gl and gG are clustered in the US (Spatz et al. 1994; Maeda et al. 1998). The glycoproteins gB (gp 143/108), gC (gp 113) and gD (gp 60) are major glycoproteins corresponding to neutralizing epitopes (Maes, Fritsch, Herr & Rota 1984; Horimoto et al. 1990). Their viral neutralizing activity is enhanced by complement supplementation. The gC serves as a receptor for complement component C3b. Binding of this complement factor may protect herpesvirus infected cells from complement-mediated lysis (Horimoto et al. 1990; Maeda et al. 1998).

In addition, gD has shown to induce hemagglutination-inhibiting antibody responses to feline RBC only and may restrict receptors of cells from felids. It also appears to be the primary candidate for any subunit vaccine against FHV-1 infection (Willemse et al. 1995; Maeda et al. 1998).
Although feline herpesviruses are genetically and antigenically closely related to canine herpesvirus (CHV) and phocid herpesvirus-1 (PhHV-1), cross-infections have not yet been reported in species other than felids (Rota, Maes & Evermann 1986a; Horimoto et al. 1990; Gaskell et al. 2006). To date, many serological comparisons made by virus neutralization tests of FHV-1 isolates have shown homogeneity among the strains (Crandell, Ganaway, Nieman & Maurer 1960; Horimoto et al. 1992; Prattelli et al. 1999).

1.2.3. Phenotypic characteristics

Feline herpesvirus type 1 field strains are generally similar with respect to pathogenicity; however, there may be slight differences between some biotypes with the existence of attenuated vaccine strains and some apparently hot challenge strains (Gaskell & Willoughby 1999; Gaskell et al. 2007). Membrane glycoproteins commonly regarded as non-essential, as their genes can be deleted from the viral genome with little or no effect on replication in vitro, are dispensable for virus multiplication in cell culture (Spatz et al. 1994). These glycoproteins are related to a variety of functions during expression in the infected cell and mediate several important biological functions; deletion of one or more of these genes can attenuate the virus (Dingwell, Brunetti, Hendricks, Tang, Tang, Rainbow & Johnson 1994; Spatz et al. 1994). They endow the virus with virulence and the ability to infect both cells in culture and a specific subset of natural host cells in vivo. They are required for FHV-1 growth in tissue culture cells, evasion of the immune response and maintenance of the virus in its natural population (Brideau, Enquist & Tirabassi 2000).

Although the virulence of FHV-1 is influenced by several genes, the gE-gI complex is a determining factor of virulence and its main function appears to be to promote cell-to-cell spread and syncytium formation during infection with FHV-1 (Dingwell et al. 1994). Phenotypically, strains with deletions in the gE gene or both gE and gI genes are attenuated, enter cells with equal efficiency, maintain their immunogenicity but

Previous studies had reported similarities among FHV-1 phenotypic characterization of various isolates. After 100 passages of the C-27 isolate (prototype of FHV-1) in primary feline cell cultures, Crandell (1971) did not observe any loss in capacity to produce severe clinical illness in kittens. Furthermore, following virus inoculation in CrFK, Horimoto et al. (1992) did not find any significant differences in growth properties between domestic isolates; plaques were similar in regard to their time of appearance, shape and size.

_Growth in cell culture_

Feline herpesvirus type 1 is generally highly cytopathic in cell cultures, and has a relatively short replicative cycle of 24 hours (Gaskell et al. 2006). The FHV-1 growth in tissue culture is accompanied by a rapid cytopathic effect (CPE) with small foci as early detectable lesions. They consist of cells with either a slight granular nucleus in which the nucleoli are small or inapparent; or cells rounded or elongated in shape and joined together by protoplasmic bands. The foci increase in size and number and are present throughout the entire monolayer. Individual cells lose its distinct cellular membrane and fuses with adjacent cells to form multinucleated giant cells, syncytia. These syncytia contract and eventually break away from cellular mass to form separate multinucleated giant cells (Crandell & Despeaux 1959; Ebner & Crandell 1960).

Histologically, in the final stage of development, these giant cells are round or club shaped and have a granular, intense eosinophilic cytoplasm with nuclei about the periphery. The presence of the intranuclear inclusions is usually associated with degenerative changes in the cytoplasm as well as condensation and margination of chromatin. In a stained preparation, this nuclear material forms a well developed
inclusion surrounded by a clear halo. Nuclear changes with well-formed inclusions are present about the time of release of new extracellular virus (Crandell & Despeaux 1959; Murphy et al. 1999).

Feline herpes virus type 1 is similar to varicella zoster virus and both remains relatively strongly cell-associated viruses with titres of two logs higher compared to viruses released in the supernatant (Herrmann et al. 1984; Gershon, Chen, LaRussa & Steinberb 2007).

1.3. Epidemiology of FHV-1 induced diseases

1.3.1. Distribution

Infection with FHV-1 has a worldwide distribution with occurrence in both domestic and wild felids. It is the most frequently detected infection of domestic cats in which it is estimated to cause 50% of all respiratory infections. In addition, 75% of cat populations are seropositive for FHV-1 antibodies (Gaskell & Willoughby 1999).

1.3.2. Hosts/Reservoirs

There are no known non-feline reservoirs or alternative hosts of FHV-1 (Gaskell et al. 2007). Feline herpesvirus type 1 is characterized by a narrow host range. Natural infections have been observed only in the members of the family Felidae and in vitro growth is limited to cells of feline origin (Povey 1979). Healthy felids that are latently infected serve as reservoirs and may shed virus when experiencing reactivation (Gaskell & Willoughby 1999).
1.3.3. Transmission

Acutely infected animals are clearly one of the most important sources of virus (Gaskell & Povey, 1982). Transmission of FHV-1 occurs mainly through direct contact between infected and susceptible animals (Radford et al. 2004).

Reactivated latent virus is likely to play an important role in FHV-1 perpetuation and spread. Latently infected animals may transmit FHV-1 to their offspring because parturition and lactation are typical stress-inducing factors leading to viral reactivation and shedding. However, transplacental infection has not yet been demonstrated in the field (Gaskell & Povey 1982).

Experimentally, other routes of transmission i.e. intravaginal and intravenous have been investigated and have produced congenital infection of kittens (Bittle & Peckham 1971) and abortion due to severe maternal rhinopneumonitis rather than foetal infection (Hoover & Griesemer 1971).

AIM AND OBJECTIVES

This study was conducted with the aim of determining the existence of possible variations in genetic, antigenic and phenotypic characteristics between FHV-1 isolates circulating among domestic and wild felids in South Africa.

The objectives of the study were:

- To determine the genetic relatedness between three FHV-1 isolates respectively from a domestic cat (Felis catus), a cheetah (Acinonyx jubatus) and an African wild cat (Felis silvestris) in South Africa.
- To determine the antigenic relatedness of the three isolates.
- To compare the phenotypic characteristics of the three isolates.
CHAPTER 2

MATERIALS AND METHODS

2.1. Cell culture and media

A seed culture of Crandell Reese feline kidney (CrFK) cells originally obtained from the American type culture collection (ATCC) was used for propagating FHV-1 isolates. The cells were grown as monolayers in flasks in minimum essential medium (MEM) supplemented with 10% foetal calf serum (FCS) (Highveld biological products), 10% tryptose phosphate broth and gentamycin (1 ml/1000 ml). Foetal calf serum was reduced to 5% in maintenance cultures. Once the monolayers had formed, the cells were trypsinized, centrifuged and resuspended in a freezing medium consisting of 60% MEM, 30% FCS and 10% dimethylsulfoxide (DMSO). Cells from a 75 cm$^2$ tissue culture flask were resuspended in 3 ml freezing media and dispensed in volumes of 1.5 ml in freezing ampoules. It was then, wrapped in cotton wool and left for 24 hours respectively at -20 °C and -70 °C before final storage in liquid nitrogen. Before use, cells were rapidly thawed for 2 min in a water bath, and then grown as a monolayer in 75 cm$^2$ flasks.

2.2. FHV-1 strains selected

The three FHV-1 isolates used in this study were obtained from the Department of Veterinary Tropical Diseases (DVTD), Faculty of Veterinary Science, University of Pretoria. The FHV-1 isolate from the cheetah was identified by means of a electron microscopy and direct fluorescent antibody test using an FHV-1 conjugate (VMRD Inc., USA) (Van Vuuren et al. 1999), whereas the identify of the FHV-1 isolate from the domestic cat and wild cat were confirmed by means of PCR (R. Bhoora, unpublished results 2004).
To prepare a virus stock, each isolate was propagated in CrFK cells. Cells were incubated in 25 cm$^2$ tissue culture flasks until confluent. Growth media was removed and 0.2 ml of each viral stock was added and allowed to adsorb for 1h at 37 °C, after which cells were washed once with MEM containing 5% FCS. Before a further incubation at 37 °C, a volume of 10 ml of fresh MEM containing 5% FCS was added to flask. When cytopathic effects (CPE) involved 90% of the monolayer, the infected cells and supernatant were harvested and stored at -80 °C. Table 1 lists the isolates included in this study and their host species.

Table 1. FHV-1 isolates, year of collection and host species

<table>
<thead>
<tr>
<th>Isolated designation</th>
<th>Year isolated</th>
<th>Host species</th>
</tr>
</thead>
<tbody>
<tr>
<td>V116/97</td>
<td>1997</td>
<td>Cheetah</td>
</tr>
<tr>
<td>V70/00</td>
<td>2000</td>
<td>Domestic cat</td>
</tr>
<tr>
<td>V15/01</td>
<td>2001</td>
<td>African wild cat</td>
</tr>
</tbody>
</table>

2.3. Preparation of viral antigen for use in serum neutralization test

The antigens were prepared from infected cell cultures and the procedure was a modification from the method described by Schmidt (1989). For preparation of serological antigen with high proportion of infectious virus; viruses were propagated under conditions giving maximum infectivity titers. The high concentration of viral antigens was achieved by infecting monolayers in a small volume of maintenance medium (Schmidt 1989). Confluent monolayers of CrFK in 75 cm$^2$ were washed twice with phosphate buffered saline with calcium and magnesium (PBS$^+$). Fresh MEM containing 5% of FCS was added just to cover the cells before being infected with 0.5 ml of the stock virus and left for 1h for viral adsorption. Maintenance media was added and infected cultures were incubated at 37 °C until 90% of the monolayer exhibited CPE. After the first passage the flasks were frozen at -80 °C, thawed once and the contents centrifuged at low speed, 6,700 g for 30 s, to remove cell debris. The supernatant was collected, aliquoted in 0.8 ml in cryotubes and stored at -80 °C until needed.
2.4. DNA extraction

2.4.1. Purification of FHV-1

To ensure that defective interfering particles which can lead to altered endonuclease restriction patterns were not present in the virus stock preparation, only DNA isolated from purified virus obtained from low multiplicity of infection (MOI) passages were used in the experiment (Henry, Robinson, Dauenhauer, Atherton, Hayward & O'Callaghan 1981; Zweerink et al. 1981). Crandell Reese feline kidney cells were grown till confluent in five 150 cm² flasks, and infected with 1 ml of each FHV-1 isolate at a low MOI of 0.01 plaque forming units (PFU) per cell. The virus was allowed to adsorb for 1 hour at 37 °C in an incubator before addition of MEM containing 5% of FCS. When CPE was advanced, infected cells were harvested and purified by ultracentrifugation through a 40% sucrose cushion. The protocol was described by Spear & Roizman (1972). Briefly, cells and medium were collected and clarified by low centrifugation (4000 g, for 40 min at 4 ºC). The pellets were kept on ice, suspended in approximately 10 ml of 2 mMTris/Triton X-100, pH 8, disrupted with strokes of a dounce homogenizer and centrifuged at 4 000 g, for 40 min at 4 ºC and repeated 3 times. For further purification, the washed pellets were resuspended in 5 ml PBS and centrifuged through a 40% sucrose cushion (w/v in 2 mM Tris pH 8) at 19 000 g, for 60 min at 4 ºC. The purified virions were collected as a white pellet in the tube and resuspended in 0.5 ml TNE buffer (10 mM Tris–HCl, 100 mM NaCl, 5 mM EDTA pH 8.0).

2.4.2 Purification and precipitation of genomic DNA

The protocol was adapted from Sambrook, Fritsch & Maniatis (1989). The suspended viral pellet was lysed using 0.5% sodium dodecyl sulfate (SDS) and protease K (Qiagen) (200 mg/ml final concentration) at 56 ºC for 2.5 hours (Deregt, Jordan, Van Den Hurk, Masri, Tessaro & Gilbert 2000). An equal volume of phenol was added to each sample and the mixture repeatedly inverted to mix. The
samples were then centrifuged at 20 800 g in a bench top centrifuge at 4 °C for 5 min. The top aqueous layer was removed and placed in a fresh microcentrifuge tube. An equal volume of phenol/chloroform (1:1) was added to each sample, and centrifuged for 5 min at maximum speed. Again the top aqueous layer was removed, an equal volume of chloroform added and centrifuged for 5 min at maximum speed. The aqueous fraction was removed and placed in a clean microcentrifuge tube. The DNA was then precipitated in two volumes of absolute ethanol with one tenth of volume 3 M NaAc (pH 5.2); 2 µl of pellet paint co-precipitant (Novagen: USA) was added to each preparation to aid precipitation of DNA. The DNA pellet was washed respectively with 70% and 100% ethanol, centrifuged at 20 800 g for 5 min. The supernatant was removed and the DNA pellets air-dried. The dried pellets were resuspended in 10 µl of TE buffer (Ambion) (Deregt et al. 2000) and the concentration determined by spectrophotometry (Nanodrop ND1000).

For further purification, the DNA suspension was extracted twice with TE-buffered phenol (Henry et al. 1981) followed by a third phenol/chloroform (1:1) extraction then precipitated with ethanol as described above.

2.5. Nested PCR

To ensure that the viruses were feline herpesviruses, the robust nested PCR method based upon amplification of the highly conserved DNA polymerase gene with published consensus degenerate primers (VanDevanter, Warrener, Bennett, Schultz, Coulter, Garber & Rose 1996; Rose 2005) (Table 2) for detection and partial identification of herpesviruses present in tissue samples and cultured cells, even when no prior DNA sequence information is available were used to confirm the identity of the isolates. An expected PCR product ranging in size from approximately 215 to 315 bp confirmed the identification of a herpesvirus (VanDevanter, et al. 1996).

Amplification of a region of the herpesvirus DNA polymerase gene was performed, with modifications, as previously described by VanDevanter et al. 1996 and Ehlers,
Brochers, Grund, Frolich, Ludwig & Buhk (1999), using consensus degenerate primers (Fermentas) targeting a highly conserved gene sequence. The first amplification of DNA (10 to 70 ng) was performed with two upstream primers, DFA and ILK, and one downstream primer, KG1 (VanDevanter et al. 1996) (Table 2). Two µl of the template DNA (10-50 ng) were mixed with 12.5 µl of master mix (Fermentas), 20 pmol of each primer and water in a total volume of 25 µl. Reactions were cycled 35 times with 30 s of denaturation at 94 °C, 60 s of annealing at 46 °C, and 3 min of strand extension at 72 °C. After cycling, the reaction mixtures were incubated for 7 min at 72 °C and were then held at 4 °C until analyzed. A positive built-in control in the form of the FHV-1 virus previously identified by electron microscopic examination of cell culture and direct fluorescent antibody test using fluorescein-conjugated FHV-1-specific antibodies (VMRD Inc., USA) (Van Vuuren et al. 1999), and deionized double distilled water was used as negative control in each run. Twenty µl of PCR products were loaded on a 2% agarose gel containing 1.5 µl/50 ml (stock 10 mg/ml) of ethidium bromide. The electrophoresis was carried out in 1xTAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8) at 110 volts for 45 min. The gel was visualized and photographed under UV light using a gel documentation system (Dual intensity Transilluminator: UVP upland, California).

The nested amplification was performed with one upstream primer, TGV, and one downstream primer, IYG (VanDevanter et al.1996) (Table 2). Reaction mixtures contained 2.5 µl of the primary PCR product as template, 20 pmol of each primer, 12.5 µl of master mix (Fermentas) and water in a total volume of 25 µl. The PCR, conditions were the same except that extension times were reduced to 60 s and the cycles increased to 45. Nested PCR products were analyzed and visualized under the same conditions used for the primary reaction.
Table 2. Sequences of consensus degenerate PCR primers

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFA</td>
<td>5’GAYTTYGCNAGYYTNTAYCC 3’</td>
</tr>
<tr>
<td>ILK</td>
<td>5’TCCCTGGACAAGCAGCARNYSGCNMTNAA3’</td>
</tr>
<tr>
<td>KG1</td>
<td>5’GTCTTGCTCACCAGNCTCNACNCYTT3’</td>
</tr>
<tr>
<td>TGV</td>
<td>5’TGTAACCTCGGTGTAYGGNTTYACNGGNGT3’</td>
</tr>
<tr>
<td>IYG</td>
<td>5’CACAGAGTCCGTRTNCRTADAT3’</td>
</tr>
</tbody>
</table>

2.6. Restriction fragment length polymorphism

2.6.1 Restriction endonuclease digestion

To determine the relatedness of the different isolates, the discriminatory power of Restriction fragment length polymorphism (RFLP) were used to differentiate between closely related strains by analyzing the restriction endonuclease cleavage patterns.

The purified DNAs were digested with the RE SalI and KpnI (Fermentas) which have been successfully used in previous studies of restriction endonuclease analysis of the FHV-1 genome and were selected as they each yielded moderate numbers of 15 and 17 fragments which could be easily interpreted (Scherba et al. 1988; Grail et al. 1991; Horimoto et al. 1992; Hamano et al. 2005).

Gene sequences from herpes simplex virus type 1, namely gE, gI, gD, and gG as well as sequences of hypothetical protein and other partial and complete coding sequences available on Genbank (accession number D42113) were also used to identify additional RE sites on the corresponding portion of the FHV-1 genome, using the Staden package gap4 program. In addition to nine HindIII restriction sites which generate fragments of sizes 552, 759, 2724, 3275, 3358, 4002, 5083, 7132 & 7982 base pairs (bp) five EcoRV and Darl restriction sites generating respectively
fragments of sizes 1145, 3338, 3721, 4129 & 7154 bp and 815, 2973, 3328, 3606 & 5070 bp were identified. The genomic DNA of the domestic cat was digested by the restriction enzymes (RE) \textit{Hind}II, \textit{EcoRV} and \textit{Dar}I prior to further purification.

A concentration of 5 \(\mu g\) in a volume of 1 \(\mu l\) of each viral DNA was digested overnight at 37 \(^\circ\)C in a thermocycler using 1.25 U (Deregt \textit{et al.} 2000), respectively with \textit{Sal}I in 1x buffer o (50 mM Tris-HCl (pH 7.5), 10 mM NaCl, 0.1 mg/ml BSA and \textit{Kpn}I in 1x buffer \textit{Kpn}I (100 mM Tris-HCl (pH 7.5), 10 mM MgCl\(_2\), 0.02% Triton x-100, 0.1 mg/ml BSA) and water to a final volume of 20 \(\mu l\).

\textbf{2.6.2. Gel electrophoresis}

A volume of 20 \(\mu l\) of the digested DNAs was subjected to electrophoresis using a 0.7% agarose gel containing 1.5 \(\mu l/50\) ml (stock 10 \(\mu g/ml\)) of ethidium bromide in 1xTAE buffer (40 mM Tris-acetate, 1mM EDTA pH 8) at 30 volts for 12 hours (Deregt \textit{et al.} 2000). The gel was visualized and photographed under UV light using a gel documentation system (Dual intensity Transilluminator: UVP upland, California). The fermentas O’gene ruler™1kb DNA ladder (Burlington Canada) was used as a molecular marker.

\textbf{2.7. Random Amplified Polymorphic DNA}

\textbf{2.7.1. Amplification reaction}

For the RAPD-PCR, 6 published (Afonso \textit{et al.} 2007) random primers (Nqaba Biotechnical) were tested (Table 3). The amplification was carried out in a 25 \(\mu l\) reaction volume consisting of 12.5 \(\mu l\) of master mix (Fermentas), 1 \(\mu l\) of primer and an amount of 10 ng in a volume of 2 \(\mu l\) of DNA from each virus and 9.5 \(\mu l\) of water. The RAPD-PCR conditions consisted for an initial denaturation step of 2 min at
95 °C, 32 cycles of 95 °C for 30 s, 37 °C for 1 min, 72 °C for 1 min and an elongation step of 72 °C for 5 min.

2.7.2. Gel electrophoresis

A volume of 5ml of the amplified product was loaded on 2% MS agarose gel containing 1.5 µl/50 ml (stock 10 µg/ml) of ethidium bromide in 1xTAE buffer (40 mM tris-acetate, 1mM EDTA pH 8) at 100 volts for 90 min. The gel was visualized and photographed under UV light using a gel documentation system (Dual intensity Transilluminator: UVP upland, California).

Table 3. Sequences of primers used in the RAPD analysis

<table>
<thead>
<tr>
<th>Primers designation</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAPD01</td>
<td>5′-GTAGACCCGT-3′</td>
</tr>
<tr>
<td>RAPD02</td>
<td>5′-AAGAGCCCCGT-3′</td>
</tr>
<tr>
<td>RAPD03</td>
<td>5′-AACGCGCAAC-3′</td>
</tr>
<tr>
<td>RAPD04</td>
<td>5′-ACCCCGAAG-3′</td>
</tr>
<tr>
<td>RAPD05</td>
<td>5′-CACACACACA-3′</td>
</tr>
<tr>
<td>OPA 05</td>
<td>5′-GGGACGTTG-3′</td>
</tr>
</tbody>
</table>

2.8. Serum neutralization test

2.8.1. Virus titration

The test was carried out in 96-well flat bottom plates with lids (AEC Amersham) as described by Pead & Holley (1973) and Schmidt 1989. Suspensions of fresh virus were obtained by passage in cell culture. Serial ten fold dilutions of each virus were prepared in tubes starting from $10^{-1}$ to $10^{-6}$. A volume of 100 µl of MEM containing 5% FCS was added in quadruplicate on the microplate. Starting at the higher virus
dilution, the same volume of virus was added to all the wells accordingly. To set up a cell control, 200 µl of MEM containing 5% FCS were added to 8 wells. Before incubation a volume of 80 µl of 480 000 cells/ml were added to all wells. The plates were placed in an incubator at 37 °C in a humid atmosphere containing 5% CO₂, and monitored daily for cytopathic effect (CPE) at intervals over five days with an inverted microscope. The control wells were checked to ensure that the virus-free cells had not deteriorated. The endpoint was calculated with the method of Reed and Muench (1938) and expressed as the 50 per cent tissue culture-infective dose (TCID₅₀) per 0.1 ml of inoculum.

2.8.2. Monoclonal antibodies

Nine monoclonal antibodies (MAbs) (Custom Monoclonals International) prepared against a vaccine strain of domestic cats were tested in a microneutralization assay against the three isolates (domestic and wild felids) to determine whether any could neutralize the in vitro infectivity of FHV-1. The MAbs were previously commercially produced. These MAbs were of the immunoglobulin G isotope and bound to various epitopes on the major glycoproteins of FHV-1. The MAbs contained 0.05% sodium azide as preservative, were all kappa light chain and had different glycoprotein targets. Each MAb was supplied at a known (or best estimate) mg/ml concentration; some were staphylococcal protein A purified, others as ammonium sulphate precipitation (NH₄)₂SO₄ or even culture supernatant (Table 4) summarizes the MAbs used in this study and their properties.
Table 4. Summary of the characteristics of the FHV-1 monoclonal antibodies.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Supplied</th>
<th>Concentration mg/ml</th>
<th>Immunoglobulin isotopes</th>
<th>Glycoprotein target</th>
</tr>
</thead>
<tbody>
<tr>
<td>FHV7-5</td>
<td>Staphylococcal protein A</td>
<td>0.75</td>
<td>IgG2B</td>
<td>gD</td>
</tr>
<tr>
<td>FHV7-7</td>
<td>Staphylococcal protein A</td>
<td>2.5</td>
<td>IgG2A</td>
<td>gD</td>
</tr>
<tr>
<td>S1-6</td>
<td>Culture supernatant</td>
<td>(est) 0.025</td>
<td>IgG2B</td>
<td>-</td>
</tr>
<tr>
<td>CCM1-3E</td>
<td>Culture supernatant</td>
<td>(est) 0.025</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CV7-6B</td>
<td>Culture supernatant</td>
<td>0.025</td>
<td>IgG2A</td>
<td>gD</td>
</tr>
<tr>
<td>CV8-11A</td>
<td>Culture supernatant</td>
<td>(est) 0.025</td>
<td>IgG2B</td>
<td>-</td>
</tr>
<tr>
<td>FHV-5</td>
<td>Staphylococcal protein A</td>
<td>0.35</td>
<td>-</td>
<td>gB &amp; gD</td>
</tr>
<tr>
<td>CV8-9A</td>
<td>(NH₄)₂SO₄</td>
<td>0.25</td>
<td>IgG2B</td>
<td>gD</td>
</tr>
<tr>
<td>CV8-10E</td>
<td>(NH₄)₂SO₄</td>
<td>(est) 0.25</td>
<td>IgG1</td>
<td>gB</td>
</tr>
</tbody>
</table>

2.8.3. Quantification of monoclonal antibodies

The MAbs were dialyzed at room temperature for 2 hours and then overnight against fresh phosphate buffered saline (0.1 M sodium phosphate, 0.15 M sodium chloride, PH 7.2) using A-Lyser dialysis cassettes (Pierce, Rockford IL, USA) and further against MEM with antibiotics (1 ml gentamycin/1000 ml MEM) for 2 hours. The concentration was then determined using a spectrophotometer (Nanodrop ND1000).
2.8.4. Reference sera

The positive control was a built-in control in the form of the FHV-1 virus previously identified by electron microscopic examination of cell culture and direct fluorescent antibody test (Van Vuuren et al. 1999). Serum from a dog used as negative reference control was obtained from the Onderstepoort Veterinary Academic Hospital (OVAH), Faculty of Veterinary Science, University of Pretoria.

2.8.5. Microneutralization assay

The serum neutralization test (SNT) was performed in 96-well flat–bottomed cell culture plates (AEC Amersham, catalogue no 167008) as described by Schmidt (1989) using the constant virus varying serum method. Initially 1:5 or 1:10 dilutions of MAb as recommended by the manufacturer (Custom Monoclonals International) were made in PBS\(^+\) and inactivated for 30 min in a 56 °C water bath. Duplicate, serial two-fold dilutions of the heat-inactivated test MAb were made from 1:2 to 1:256 and mixed with equal volumes (100 µl) of 100TCID\(_{50}\) titre of virus antigen. A built-in positive control in the form of the FHV-1 virus and a negative reference serum control from a dog were diluted to 1:5 in PBS\(^+\), and inactivated at 56 °C in water bath for 30 min. A serial two-fold dilution of the control sera was prepared and an equal volume of 100 µl of virus was added in 8 wells. As cell control, 200 µl of MEM containing 5% FCS was added to 6 wells. For virus control, a back titration of the virus suspension and four ten-fold dilutions of antigen 100TCID\(_{50}\) were made in triplicate. A volume of 100 µl of each dilution was added to an equal volume of MEM containing 5% of FCS. The microtitre plates were incubated for 1 hour at 37 °C in an atmosphere of 5% CO\(_2\) before the addition in all the wells of 80 µl of CrFK cell suspension at a concentration of 480 000 cells/ml. The plates were read on the third day, and the virus neutralization titre was determined on the basis of cytopathic changes; the absence of CPE was an indication of neutralization (Pead & Holley 1973). The monoclonal antibody titre was expressed as the reciprocal of the highest dilution of MAb that resulted in total inhibition of CPE (Deregt et al. 2000)
2.9. Plaque production

Plaque assays were undertaken using monolayers of CrFK cells in 6-well cell culture plates as described by Bidawid, Malik, Adegbunrin, Sattar & Farber (2003). Three ml of the cell suspension containing $4.8 \times 10^5$ CrFK cells/ml were seeded in 6-well tissue culture plates; incubation for 12-16 hours at 37 °C in an humidified atmosphere containing 5% CO$_2$ resulted in a confluent monolayer. The growth media was aspirated and 500 µl of ten-fold serial dilutions of each virus prepared in MEM without serum, from $10^{-3}$ to $10^{-7}$, were inoculated separately onto monolayers. As a control, a volume of 3 ml of MEM containing 5% of FCS was added in the 6th well. Cultures were incubated for 60 min at 37 °C in a humidified atmosphere containing 5% CO$_2$.

A 2 x supplemented MEM (Celtic) overlay solution containing 10% FCS, 3% NaHCO$_3$ (7.5%) and 0.2% antibiotics was prepared during viral adsorption and kept in a water bath at 43 °C. The 1.2% agarose solution was prepared by dissolving 1.2 g of ultra purified agarose 1320 (batch 99433; cat no A1201) in 100 ml deionized distilled water and was sterilized by autoclaving for 45 min at 121 °C. This solution was also held in a water bath at 43 °C. The final overlay medium was prepared by mixing equal volumes of the 2 x supplemented MEM and the 1.2% agarose. Each monolayer received 2 ml of this mixture. The plates were left at RT for 20 min until the agarose solidified, and then incubated at 37 °C in a humidified 5% CO$_2$ atmosphere for 5 days. The cells were stained by addition of 2 ml of a second overlay of the same composition as the first, with the exception that it also contained a 1:10 000 solution of 3% neutral red. Cultures were incubated overnight at 37 °C in a humidified atmosphere containing 5% CO$_2$ and plates were examined for plaques (white areas of virus infected cells which did not take up the neutral red) (Schmidt 1989).
CHAPTER 3

RESULTS

3.1. Genetic analysis

3.1.1. Nested PCR

The PCR products of 250 bp were obtained only with the nested PCR. The bands detected on 2% agarose gel were clearly in accordance with the expected result, namely a PCR product ranging in size from approximately 215 to 315 bp (Fig. 3.1). The consensus degenerate primers DFA, ILK, TGV, IYG, and KG1 developed for herpesvirus screening when no prior DNA sequence information is available were found to amplify the region of DNA polymerase gene of all the isolates and therefore established that all the strains were herpesviruses.
Fig. 3.1 A & B. The 250 bp amplified products obtained in the nested PCR for identification of herpesvirus. M: molecular marker, lane 1: V116/79 (cheetah), lane 2: V70/00 (domestic cat), lane 3: V15/01 (African wild cat), lane 4: Negative control and lane 5: Positive control
3.1.2. Restriction fragment length polymorphism

In the restriction enzyme analysis of the DNA of the three isolates, the domestic cat genome did not appear to be cleaved by the RE \textit{Sal}I and \textit{Kpn}I. However, all these enzymes cleaved the wild felid DNA and allowed differentiation between them (Fig. 3.2). With the use of \textit{Sal}I, minor variations were detected by an additional fragment with a slower electrophoretic mobility for the cheetah (lane 1) and a more intense fragment for the African wild cat (lane 3) indicated by the arrow A; and two slight shifts of fragment mobility (lane 3) as indicated by the arrows. The RE \textit{Kpn}I generated more fragments without any loss or gain of fragments. The cleavage patterns of the genomic DNA of the Cheetah (lane 1) and the African wild cat (lane 3) were very similar except for a slight shift in the fragment mobility (lane 3) which might be attributed to the electrophoresis process as indicated by the molecular markers. The electrophoresis of the digested DNA was run in duplicate and has shown similar results during several attempts.

![Fig.3.2. Restriction endonuclease digestion patterns of FHV-1 isolates with RE \textit{Sal}I and \textit{Kpn}I. M: molecular marker, Lane 1 & 3: cheetah & African wild cat, Lane 2: domestic cat isolate.](image)
The digestion of the genomic DNA of the domestic cat virus with the RE HindII, EcoRV and DarI prior to further purification did not generate any bands (results not shown).

### 3.1.3. Random amplified polymorphic DNA

Based on the number and the distribution of different sizes of amplicons, only the primers RAPD 01, RAPD 02, RAPD 03 gave visible bands (Fig. 3.3). The RAPD assay gave a series of discrete DNA fragments. Variation in the number of fragments was higher among different primers than among different strains. RAPD analysis with primers 01 did not discriminate the strains. The patterns amplified showed conserved fragments between the strains and no obvious differences were observed in the respective electrophoresis profiles. RAPD 02 displayed an extra fragment in the African wild cat DNA, indicated by an arrow in lane 8. The profile pattern of the cheetah virus (lane 6) and the domestic cat virus (lane 7) appear to be similar. RAPD 03 showed a difference in intensity of fragments between wild and domestic cat isolates and a loss of a fragment indicated by an arrow in lane 10 (domestic cat).

![Fig. 3.3. Electrophoresis of the RAPD-PCR of three isolates on a 2% MS agarose gel. Lane 1: Molecular marker; Lane 2: negative control (cell culture). Lane 3-5: Primer RAPD 01 fingerprints; Lane 6-8: Primer RAPD](image)
02 fingerprints; Lane 9-11: Primer RAPD 03 fingerprints. Lanes 3, 6, 9 (V116/97) cheetah and lanes 5, 8, 11 (V15/01) African wild cat isolates; lanes 4, 7, 10 (V70/00) domestic cat isolate.

3.2. Serum neutralization test

3.2.1. Virus titration

The results indicated an identical 100TCID₅₀ expressed at the 10⁻⁴ dilution for the domestic isolate (V70/00) and the African wild cat (V15/01); and at 10⁻⁵ for the cheetah (V116/79).

3.2.2. Reactivity of monoclonal antibodies with FHV-1 isolates.

Only two monoclonal antibodies, CV8-9A and CV8-10 directed respectively towards glycoproteins gD and gB, inhibited the CPE of V116/97 and V15/2001 strains completely, both from wild felids, at the same antibody titre of 80 (Table 5). These MAbs specific to domestic cat FHV-1 reacted with wild felid isolates indicating that they are serologically closely related. An effect similar to a toxic effect was observed in the cell cultures when these two monoclonal antibodies reacted with the domestic cat isolate (V70/00). However, no contamination was observed in the virus control (back titration) and cell control. In order to address the problem of toxicity, attempts were made to dilute the two monoclonal antibodies CV8-9A and CV8-10E more than 1:10 with PBS⁺ followed by inactivation and repeating the assay. Secondly, the MAbs were dialyzed as they all contained 0.05 sodium azide as preservative; their concentration decreased significantly as indicate in Table 6. Despite these attempts, the “toxicity” effect could not be eliminated.
<table>
<thead>
<tr>
<th>Monoclonal Antibodies</th>
<th>Specificity</th>
<th>Immunoglobulin</th>
<th>Neutralizing titre</th>
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<tr>
<td></td>
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<td>IgG2B</td>
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<td>gD</td>
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<td>-</td>
<td>IgG2B</td>
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<tr>
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<td>gD</td>
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</tr>
<tr>
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<td>FHV-5</td>
<td>gB &amp; gD</td>
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<td>-</td>
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<tr>
<td>CV8-10E</td>
<td>gB</td>
<td>IgG1</td>
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Table 6. Monoclonal antibody concentrations after dialysis

<table>
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<th>Concentration after dialysis mg/ml</th>
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<td>FHV 7-7</td>
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<td>0.12</td>
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3.3 Phenotypic analysis

Plaques were produced using varying concentrations of virus; dilutions of $10^{-3}$ to $10^{-7}$ on CrFK monolayers. They were evident in CrFK cultures overlaid by agar on the sixth day following inoculation; further incubation resulted in enlargement of plaques. These plaques were visible to the naked eye, and could be enumerated without further magnification (Fig. 3.4.A, B and C). Isolated individual plaques were clearly visible in wells at the virus dilution of $10^{-6}$ for the wild isolates (Fig. 3.4.A and Fig. 3.4.C) and $10^{-5}$ for the domestic cat (Fig. 3.4.B). The plaques were circular in shaped and uniform in morphology. However, at lower dilutions ($10^{-3}$-$10^{-5}$) plaques became confluent or greater overlapping occurred with adjacent plaques. The difference in plaque sizes of the different strains was macroscopically visible. The domestic cat isolate (Fig. 3.4.B) produced larger plaques than those of the wild strains although its 100TCID$_{50}$ was identical to that of the African wild cat isolate. The domestic cat virus could be readily distinguished by its size from the wild isolates with the naked eye.
Fig. 3.4.A. Plaque production in CrFK cell culture with the V116/97 isolate (cheetah)

Fig. 3.4.B. Plaque production in CrFK cell culture with the V70/00 isolate (domestic cat)
Fig.3.4.C. Plaque production in CrFK cell culture with the V15/01 isolate (African wild cat)
4.1. Introduction

In this study, the genetic relatedness of FHV-1 isolates from domestic and wild felids were investigated by comparing patterns of genomic DNA cleaved with RE SalI and KpnI and the DNA fingerprints generated by different RAPD primers. A panel of nine Mabs was used in a SNT to determine the serological relationship between the isolates. In addition, the phenotypic characteristics of the isolates were also compared by their ability to produce plaques in CrFK monolayers.

4.2. Genetic comparison

PCR results were poor when samples were tested with the first set of primers (results not shown). In spite of several attempts to optimize the test: variations of the concentration of the different reagents used, template DNA concentration i.e. 2.5 µg DNA dilution from 10^{-1} to 10^{-3} fold, thermocycling temperature; results remained unsatisfactory. The optimized DNA concentration of 10-70 ng for the first PCR and the volume of 2.5 µl PCR product used in the nested amplification gave good results. Targeting conserved region of herpesvirus DNA polymerase by PCR with consensus degenerate primers has been reported to have the potential to detect the majority of existing herpesviruses (VanDevanter et al. 1996; Ehlers et al. 1999), the results of the nested PCR confirmed the identity of the isolates as herpesviruses since herpesviruses are known to undergo drastic alterations in its DNA composition when passaged at a high multiplicity of infection, only DNA of purified virions from cells infected with 0.01 PFU were used (Bronson, Dreesman, Biswal & Benyesh-
Melnick 1973). An insufficient yield of DNA of FHV-1 from columns using the midi kit (Qiagen) motivated us to attempt the phenol/chloroform extraction method. The total amount of DNA received from the infected cells (approximately 5 µg/µl) was sufficient to perform the restriction endonuclease digestion.

The domestic cat genome did not appear to be cleaved by the RE SalI and KpnI, which generated fragments in previous studies (Scherba et al. 1988; Horimoto et al. 1992; Hamano et al. 2005); nevertheless, these enzymes allowed the differentiation between the wild isolates. Minor variations were noted when comparing the restriction enzyme profiles of the wild isolates. However, when RE SalI was used it should be mentioned that the additional fragment with a slower electrophoretic mobility observed in lane 1 (Cheetah) was closer. The occurrence of an intense band in lane 3 arrow A (African wild cat) could be attributed to partial restriction digestion during RFLP analysis and might be separated if the gel was allowed to run for a longer period of time. The slight mobility shifts of fragments observed in lane 3 (African wild cat) when SalI and KpnI were used may be due to the electrophoresis process as clearly indicated by the molecular markers. The results revealed that the isolates were genetically different but confirmed a high degree of similarity between them. These findings agree with previous studies that compared domestic cat isolates of FHV-1 (Grail & Harbour 1990; Horimoto et al. 1992).

Since SalI and KpnI did not cleave the genome of the domestic cat virus, gene sequences from herpes simplex virus type 1, namely gE, gI, gD, and gG as well as sequences of hypothetical protein and other partial and complete coding sequences available on Genbank (accession number D42113) were used to identify additional RE sites on the corresponding portion of the FHV-1 genome, using the Staden package gap4 program. In addition to nine HindIII restriction sites, we were able to identified five EcoRV and five DarI restriction sites. Prior to digestion, the genomic DNA of the domestic isolate virus (V70/00) was further purified as described previously in Materials and Methods. Despite several attempts, the result remained unsatisfactory (results not shown); the genome was not cleaved by any of these restriction enzymes.
Previously, a lack of DNA digestion was reported for a bovine herpesvirus type 1 isolate from a wild ruminant (Deregt et al. 2000). Feline herpesvirus is a large DNA virus of 134 Kb and mutations occur sporadically although they may not be readily detected (Rota et al. 1986b; Grail et al. 1991, Horimoto et al. 1992). Notwithstanding mutations due to genetic rearrangements in the gC gene and differences in the geographic origin of isolates reported previously by Hamano et al. (2004 & 2005), the genomic DNAs were cleaved by RE and these were expressed by a gain or loss of fragments. Therefore, it was not possible to make a comparison due to lack of digestion of the genomic DNA of the domestic cat isolate.

In this study, RAPD analysis, a molecular technique introduced to study relationships of closely related viruses, seemed to be promising for genetic comparison. Practical problems associated with RAPD-PCR have been mostly attributed to its lack of reproducibility. Some authors (Meunier & Grimont 1993; Tyler, Wang, Tyler & Johnson 1997) believe that it remains a comparative typing technique useful for the identification of epidemiological relationships. When Stemmler, Neubauer & Meyer (2001) applied the RAPD-PCR this showed a great accuracy for analysis of closely related orthopoxviruses. The advantage of RFLP, however, is that even a point mutation can be identified, which can result in easily detectable size variation or lack of bands if the mutation affects the recognition site of the enzyme used (Stemmler et al. 2001).

In order to select RAPD primers that could be suitable to discriminate the three isolates, initial analysis were performed with all six RAPD primers provided in the kit. Depending on the primer used, different numbers of various sizes of PCR fragments were obtained (data not shown). This is not surprising since different RAPD primers amplified different regions of the DNA randomly (Ho, Phang & Pang 1995).

The RFLP pattern can be traced for the recognition sites of restriction enzyme; whereas with RAPD-PCR, it is necessary to assay a high number of primers. Primers giving poor band resolution or low or high numbers of bands are not suitable for discrimination of the isolates and therefore are rejected. An increase in the
number of bands might result in misinterpretation (Stemmler et al. 2001). The comparison of the profile patterns generated by three isolates revealed an identical banding pattern using RAPD primer 01. This result suggested that either this primer amplified a conserved region (Ho et al. 1995), or that the three isolates under investigation were genetically identical. However, using RAPD primer 03, different banding patterns were observed between the wild felid isolates on the one hand and the domestic cat isolate on the other hand. In addition, differences were observed among the wild cat isolates when RAPD primer 02 was used. The combined results of these three RAPD primers suggested that the three FHV-1 isolates used in this study were genetically different yet demonstrated a degree of similarity.

The results obtained by RLFP and RAPD analysis were very similar and the results by RAPD fingerprint analysis of the wild isolates were in agreement with the RFLP results. Both allowed discrimination between individual isolates and revealed minor variations in fragment sizes.

4.3. Antigenic comparison

In this study, a panel of nine MAbs with neutralizing activity against a domestic cat FHV-1 strain was used to investigate the serological relationship between the domestic cat virus and the two wild felid viruses. Seven of the nine MAbs did not neutralize any of the three isolates used. Previously, Pereira, Dondero, Gallo, Devlin & Woodie (1982) reported that the failure of some MAbs to react with a particular herpes simplex virus was due to the loss or modification of the antigen site. Neutralization without any antigenic variation was observed only with two MAbs, CV8-9A and CV8-10E against the wild isolates; a toxic effect on cells was observed when the domestic cat virus was used. Despite the dilution and a further purification of the MAbs by dialysis, the SNT with the domestic isolate did not give satisfactory results. The limited volumes of the MAbs due to the high cost did not allow further investigation. Since the reaction remained clear and no turbidity was observed after seven days, the toxic effect could perhaps be attributed to contamination by a slow growing organism, most likely yeast, causing subtle alteration in the MAbs behaviour.
With regard to the antigens involved in the serum neutralization reaction, it would seem reasonable to accept that the isolates belong to the same group without necessarily having total antigenic identity (Povey 1974; Holland, Marlin, Levine & Glorioso 1983). The results obtained, suggested that the epitopes defined by gD and gB might be common, functionally related or share the same immunodominant antigens (Tohya, Masuoka, Takahashi & Mikami 1991).

It is interesting to note that these MAbs, raised against FHV-1 from a domestic cat, neutralized the wild isolates suggesting that important neutralizing epitopes are shared between these viruses; thus providing the evidence for a closer evolutionary relationship. At least two antigenic determinants are shared with the wild FHV-1 as determined by the use of the domestic cat FHV-1 specific MAbs. Similar results have been reported by Deregt et al. (2000) in the antigenic comparison of elk herpesvirus type 1 with bovine herpesvirus type 1. Moreover, the results showed intimate serological and antigenic relationships among the FHV-1 viruses suggesting that the FHV-1 from domestic cat and the strains from the two wild felids used in this study belong to the same serotype. This has also been recently reported by Ali & Lila (2005) in the study on serologic relationship among Aujeszky’s disease virus isolates. The findings in this study agree with previous reports showing comparative antigenic homogeneity between domestic and wild felid FHV-1 isolates (Sherba et al. 1988, Pratelli et al. 1999). In addition, Crandell et al. 1960 and Mochizuki, Konishi & Ogata (1977) documented that all FHV-1 strains belong to one serotype.

4.4. Phenotypic comparison

Plaque production was the criterion used to determine the growth properties of each isolate in CrFK monolayers. Different protocols were compared for the production of plaques. Since the crystal violet staining procedure requires a washing step to remove the overlaid agarose which may damage the monolayer and thus resulting in variations in plaque quantification, the neutral red staining was the alternative choice. Following virus inoculation onto monolayers in a six-well microplate at a multiplicity
of 0.01 PFU per cell, the plaque forming ability of the domestic and wild isolates in CrFK was tested.

To prevent the fragility and tendency of the CrFK to peel off the plastic surface of the wells, many trials with different densities of cells per ml ranging from \(3.8 \times 10^5\) to \(5 \times 10^5\) to determine the appropriate concentration of cells were made. The results (data not shown) indicated that \(4.5 \times 10^5\) cells per ml resulted in stabilizing cell adherence to the wells with complete coverage of monolayers after 12-16 hours of incubation and production of clearly visible and well-defined plaques. Higher cell densities did not produce any plaques, whereas lower cell concentrations produced undefined zones of cell destruction. The passage level of cells and viruses were the same for all experiments.

The attempt to produce plaques using mixtures of agarose-Earle’s solution and MEM in the overlay agar remained unsatisfactory. The overlay agar prepared with 2 x MEM and 1.2% of agarose in double distilled water was effective for plaque development. This was clearly related to the formulation of the different media. The non-availability of standardized plaque assays due to the use of different cell cultures, media formulation and cell-support surfaces was previously reported (Ormerod & Jarrett 1978; Doultree, Druce, Birch, Bowden & Marshall 1999). The cell density and the lapse of time between seeding of cells and the inoculation of virus suspension are important factors for the production of plaques. This has been similarly observed by Siegl & Kronauer (1980) and Bidawid et al. (2003).

Based on the results of this study, plaques appeared at the same time (six days post-infection) and had a defined circular shape. Similar results were obtained in previous studies (Crandell et al. 1960 and Horimoto et al. 1992). However, differences were observed in plaque sizes when domestic cat and wild felid isolates were compared. In this study, larger plaques were obtained with the domestic cat isolate. Horimoto et al. (1992) in their study on comparison of FHV-1 strains of domestic cats related the production of large plaques to the adaptation of the strains
to the cell culture. Nevertheless, Gaskell, Dennis, Goddard, Cocker & Wills (1985) observed that the difference in the sizes of plaques of FHV-1 might reflect some adaptation to latency.

Previously, variation in plaque sizes related to the virulence of viruses has been reported by Sussman et al. (1995) and Kruger et al. (1996). *In vitro* analysis of feline herpesvirus type 1 recombinant with deletions in the gE gene or both gE-gI genes has shown that the mutant maintains its immunogenicity but produces very small plaques in infected cells. This is attributed to a lower ability of mutant virus to spread by direct cell-to-cell transmission. Similarly, variation in plaque size of parvovirus has been described by Carmichael (1999) with predominantly small plaques observed with attenuated strains used for vaccine production after several passages in cell culture.

However, *in vitro* phenotypes of caliciviruses have been reported to correlate with the virulence of the strain; large plaques were obtained with virus strains exhibiting high virulence compared to those of low virulence (Ossiboff, Sheh, Shorton, Pesavento & Parker 2007). This aspect should be investigated in a study of the growth kinetics of FHV-1 isolates from domestic and wild felids that differ in virulence; this may give some suggestions for the elucidation of FHV-1 phenotypes as no data is currently available.

### 4.5. Implications of the laboratory findings on the epidemiology and control of FHV-1

Although, in this study, no history of the possible association of wild and domestic felids from which the viruses were obtained was available, infection through close contact between domestic or feral cats have been assumed to be the source of the viruses for wild felids in captivity in Brazil (Batista, Vicentini, Franco, Spilki, Silvia Adania & Roehe 2005). This cross-infection between domestic and wild felids likely confirms the high degree of comparative homogeneity.
The finding that the MAbs specific for a domestic cat FHV-1 reacted with wild isolates indicates that FHV-1 vaccines and diagnostic tests used for domestic cats can also be used for wild cats in captivity.

4.6. Conclusion and recommendations.

This study is the first comparing the genetic, antigenic and phenotypic relatedness of herpesviruses from domestic and wild felids in South Africa. The relationship based on the genetic and phenotypic characterization agreed well and both indicated that the viruses from domestic and the wild felids are different but have a high degree of similarity. These differences are even smaller in terms of the antigenic relationship which is for practical purposes identical. Although restriction endonuclease and RAPD-PCR analysis have shown to be an effective means to differentiate closely related strains and have the potential to trace isolate relationships particularly for epidemiological purposes, a comparative study with several isolates including sequencing is necessary to determine the extent of their genetic relatedness.
REFERENCES


