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INVESTIGATION OF THE GAMMAHERPESVIRUS CARRIER STATUS OF BLACK WILDEBEEST (*CONNOCHAETES GNOU*)

By

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE MASTERS DEGREE IN VETERINARY MEDICINE (WILDLIFE DISEASES)

MMEDVET (FER)

DEPARTMENT OF VETERINARY TROPICAL DISEASES
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DATE SUBMITTED: JULY 2007

ACKNOWLEDGMENTS

I wish to thank Free State Nature Conservation for all their help during the culling season of 2005. The culling team and reserve managers went out of their way to help and support me while collecting samples. Without their willingness to help I would not have been able to collect all the necessary samples and especially the large number of samples that I needed.

Savvas Vrahimis helped getting the project through the red tape and Johan Watson helped with both the organizing and sampling. Frans Marais, head of the culling team for more than 15 years, helped more than he realised, especially his sense of humour! Special thanks also to Francois van den Berg (Reserve manager, Tussen-die-Riviere Nature Reserve) who helped during all the culling operations tremendously, as well as all the other reserve managers and personnel of Koppies, Maria Moroka, Gariep Dam and Sandveld Nature Reserves. I appreciate all the help and friendliness of everybody who was involved with the culling operations.

My utmost gratitude to everybody who almost froze to death during the Free State winter nights, helping me to collect samples during the culling operations. Especially my sister, Liezl Pretorius, and Hans Kooy spent many nights assisting me.

Special thanks will have to go to Hans Kooy who, with his capture team, Thaba Manzi Wildlife Services, helped tremendously, making it possible to collect samples from black wildebeest captured by his team. His support during the past few years of my studies and especially with my project have been invaluable. Without his support I probably would not have survived!

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LIST OF ABBREVIATIONS

AlHV – 1: A*lcelaphine herpesvirus type 1*

AlHV – 2: *Alcelaphine herpesvirus* 2

CI-ELISA: Competitive inhibition enzyme-linked immunosorbent assay

CpHV – 2: *Caprine herpesvirus 2*

DNA: Deoxyribonucleic acid

dsDNA: Double-stranded deoxyribonucleic acid

EDTA: Ethylenediamine tetra-acetic acid

ELISA: Enzyme-linked immunosorbent assay

FRET: Fluorescent resonance energy transfer

HipHV – 1: *Hippotragine herpesvirus* 1

ICTV: The International Committee on Taxonomy of Viruses

ImHV – 1: *Impala herpesvirus* 1

MAb: Monoclonal antibody

MCF: Malignant catarrhal fever

MCFV: Malignant catarrhal fever virus

MCFV-WTD: Malignant catarrhal fever virus – white-tailed deer

ORF: Open reading frame

OvHV – 2: O*vine herpesvirus type 2*

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

RuRV: Ruminant Rhadinovirus

SpHV – 1: *Springbok herpesvirus* 1

SpHV – 2: *Springbok herpesvirus* 2

$T_{m:}$ Melting temperature

SUMMARY

INVESTIGATION OF THE GAMMAHERPESVIRUS CARRIER STATUS OF BLACK WILDEBEEST (*CONNOCHAETES GNOU*)

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Malignant catarrhal fever (MCF) is a mostly fatal lymphoproliferative disease in cattle, pigs, farmed deer, bison and a range of wild ungulates in zoos and game parks. The disease in domestic cattle is caused by either *alcelaphine herpesvirus type 1* (AlHV–1) or *ovine herpesvirus type 2* (OvHV–2). Both are members of the subfamily *Gammaherpesvirinae*. The major reservoir host of AlHV–1 is the blue wildebeest (*Connochaetes taurinus*), but it is generally accepted that the black wildebeest (*Connochaetes gnou*) is also a reservoir host. Sheep is the reservoir host for OvHV–2. These viruses are non-pathogenic in their natural hosts. No viral studies in the black wildebeest have been reported and as the carrier status of black wildebeest has not been documented, samples from 304 black wildebeest and 51 of their foetuses were collected for this purpose. Blood samples, including serum and blood collected in EDTA-coated collection tubes; cornea and spleen samples were collected from culled black wildebeest. Cornea and spleen samples were collected from foetuses during the culling operations. Blood samples, as above, were also collected from live animals during the capture of

such animals. Tissue and EDTA-blood samples were tested by means of conventional and real-time polymerase chain reaction (PCR) assays for detection of a gammaherpesvirus similar or related to AlHV–1. Conventional PCR failed to produce any consistent results. Real-time PCR successfully amplified a region on the gene that codes for a transactivator protein, open reading frame (ORF) 50. Melting curves were generated to determine which samples were positive for a gammaherpesvirus. Only 15.8% of the animals tested positive with the real-time PCR assay. Ninety percent (90%) of the foetuses tested positive and suggests that, unlike sheep lambs, the virus is mainly transmitted *in utero* and soon after birth. Virus isolated from a black wildebeest calf of one week of age, was confirmed by electron microscopy and sequence analysis to be a gammaherpesvirus related to AlHV–1 and used as a positive control for the real-time PCR assays. Serum samples were tested by a direct competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA) for group specific malignant catarrhal fever virus antibody. All the serum samples that were tested of culled and live animals, tested positive with the CI-ELISA. This indicates a persistent infection and a carrier status.

Chapter 1

INTRODUCTION

1.1 PREAMBLE

The family *Herpesviridae* (herpesviruses) comprises a large group of viruses that can cause widespread disease in humans, domestic and wild animals and appears to be the most common virus found in wild animals[47]. These viruses cause lifelong infections and persist in the body in a latent form in especially sites such as nerve ganglia and lymphocytes[47]. Members of the subfamily *Gammaherpesvirinae* predominantly infect lymphocytes[14] and contains two genera of lymphotropic viruses, *Lymphocryptovirus* and *Rhadinovirus* (Table 1.1)[26].

The subfamily *Gammaherpesvirinae*, genus *Rhadinovirus,* are double-stranded deoxyribonucleic acid (DNA) viruses that are typically enveloped, icosahedral and highly cell-associated[16]. According to the phylogenetic analysis of the currently known ruminant rhadinoviruses, the group appears to comprise three distinct genetic lineages (Table 1.1, Figure 1.1). The first group is the malignant catarrhal fever (MCF) subgroup, which is defined by the sequence identity and presence of the 15-A epitope. This subgroup consists of nine viruses of which four viruses are associated with the disease. The second subgroup contains the previously reported bovine lymphotropic herpesvirus and related viruses and is devoid of the 15-A epitope. *Bovine herpesvirus 4* represents the third subgroup. A phylogenetic tree of the bovine rhadinoviruses based on the amino acid sequence of the DNA polymerase gene is shown in Figure 1.1[26].

Table 1.1: Summary of the virus family *Herpesviridae* (herpesviruses)[22]

Subfamily	Genus	Examples of virus species
Alphaherpesvirinae		
Betaherpesvirinae		
Gammaherpesvirinae		
	Lymphocryptovirus	*Cercopithecine herpesvirus 12, 14 & 15*
		Human herpesvirus 4
		Callitrichine herpesvirus 3
		Pongine herpesvirus 1, 2 & 3
	Rhadinovirus	*Alcelaphine herpesvirus 1*
		Alcelaphine herpesvirus 2
		Ovine herpesvirus 2
	Subgroup 1	*Hippotragine herpesvirus 1*
		Caprine herpesvirus 2
		Malignant catarrhal fever virus – white tailed deer (MCFV – WTD)
		Equid herpesvirus 2, 5 & 7
		Ateline herpesvirus 2
		Murid herpesvirus 4
	Subgroup 2	*Mustelid herpesvirus 1*
		Saimiriine herpesvirus 2
		Cercopithecine herpesvirus 17
		Human herpesvirus 8
	Subgroup 3	*Bovine herpesvirus 4*

Note: The International Committee on Taxonomy of Viruses (ICTV) oversees the naming and classification of viruses. Viral classification starts at the level of order (-virales), then family (-viridae), subfamily (-virinae), genus (-virus) and species (-virus). The recognition of orders is very recent and therefore not yet complete. Website: www.ncbi.nlm.nih.gov/ICTVdb/

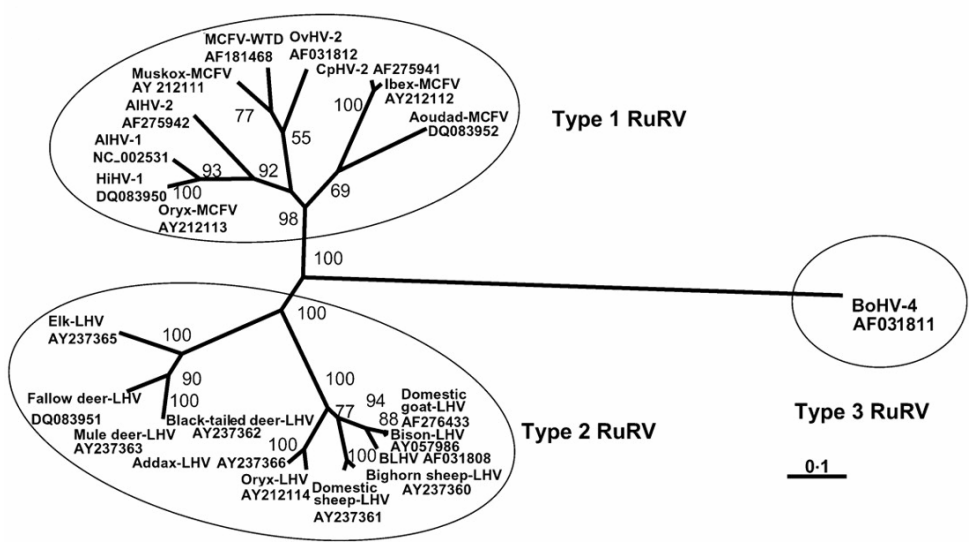

Figure 1.1: Phylogenetic tree of ruminant rhadinoviruses based on the amino acid sequences of DNA polymerase gene fragments. GenBank accession numbers for the sequences are shown on the tree and bootstrap values are shown at branch points[26].

1.2 MALIGNANT CATARRHAL FEVER

Malignant catarrhal fever in cattle and several other wild ruminants is a fatal lymphoproliferative disease. The incubation period is very difficult to determine but can range from two weeks to nine months. The clinical signs in order of occurrence, include a sudden, persistent pyrexia and generalized lymphadenopathy; severe congestion, necrosis and erosion of the nasal and oral mucosae; mucopurulent nasal and ocular discharges with excessive salivation; scleral and conjunctival congestion; centripetal corneal opacity; an iridocyclitis with hypopyon; central nervous signs as a result of underlying meningo-encephalomyelitis with muscle tremors, incoordination, head pressing, nystagmus, ear twitching, torticollis and even aggressive behaviour; diarrhoea or dysentery; as well as dermatitis and laminitis occasionally[50].

1.3 RESERVOIR HOSTS

Malignant catarrhal fever is a highly fatal and economically important disease of mainly domestic cattle caused by either *alcelaphine herpesvirus type* 1 (AlHV–1) or *ovine herpesvirus type* 2 (OvHV–2). Both are members of the subfamily *Gammaherpesvirinae*, genus *Rhadinovirus*. The major reservoir host of AlHV–1 is the blue wildebeest (*Connochaetes taurinus*), but it is generally accepted that the black wildebeest (*Connochaetes gnou*) is also a reservoir host. Most of the documented research studies regarding the role of the reservoir host in Africa have focused on the blue wildebeest. Serological studies have indicated, however, that the black wildebeest is infected with a virus antigenically related to AlHV–1 found in blue wildebeest[2] and that the virus is also responsible for MCF in domestic cattle and several other antelope species[56]. These viruses produce no clinical disease in their natural host species. Domestic sheep is the reservoir host of OvHV–2.

Recently two other pathogenic viruses have been identified that are able to cause MCF in deer. *Caprine herpesvirus* 2 (CpHV–2) is endemic in domestic goats[26]. Goats, however, can be carriers of both OvHV–2 and CpHV–2[27]. Malignant catarrhal fever in deer caused by CpHV–2 seems to be a more chronic disease and somewhat less virulent than when caused by OvHV–2[27]. The other virus causes classical MCF in white-tailed deer but the reservoir species has not been identified and the virus is therefore tentatively called malignant catarrhal fever virus – white-tailed deer (MCFV–WTD)[25,26].

1.4 SUSCEPTIBLE SPECIES

Alcelaphine herpesvirus type 1 causes disease in Africa where wildebeest graze with or in the near vicinity of cattle[3,4,5], as well as

worldwide in zoological parks where wildebeest is housed in close association with other ruminant species[40,41,62,66,67]. Species from the family *Cervidae* seem to be very susceptible to MCF, especially Pére David's deer (*Elaphurus davidianus*), red deer (*Cervus elaphus*), axis deer (*Axis axis*), rusa deer (*Cervus timorensis*) and sika deer (*Cervus nippon*). Asiatic wild cattle, such as the banteng (*Bos javanicus*) and the Indian gaur (*Bos gaurus gaurus*) and the North American and European bison (*Bison bison* and *B. bonasus*) are also very susceptible[46,61]. Kudu (*Tragelaphus strepsiceros*), sitatunga (*Tragelaphus spekei*) and Asian nilgai (*Boselaphus tragocamelus*) are also reported to be susceptible[16,24,28,50]. The sheep agent, OvHV–2, occurs worldwide, normally causing sporadic disease in cattle and pigs[32] though it can occasionally cause epizootics. *Bos indicus* and *Bos taurus* cattle are more resistant to the sheep-associated form of the disease than the wildebeest-associated form. In Asian countries, the problem is particularly acute as some local cattle (e.g. Bali cattle in Indonesia) are much more susceptible to the disease than other bovine species[11,20,21,70,71].

1.5 PATHOGENESIS

The disease caused by both AlHV–1 and OvHV–2 is very similar. It is characterised by a T-lymphocyte hyperplasia in lymphoid organs and the accumulation of these cells in non-lymphoid tissues; degeneration, necrosis and hyperkeratosis of epithelial surfaces; and a generalised, fibrinoid vasculitis[30,31,56]. There is a noticeable absence of any evidence of virus replication within these lesions as viral antigen and nucleic acid appears to be present in a limited number of cells. It has been proposed that the pathogenesis of this disease is related to an immune reaction similar to a graft-versus-host reaction. It is suggested that a virus-induced cytokine cascade results in activation of auto-aggressive T-lymphocytes, either directly

through clonal stimulation or by depression of specific suppressor cell populations. This leads to tissue damage through lymphokine-activated killer cells[56].

1.6 RELATED GAMMAHERPESVIRUSES

Serological studies have shown the presence of antibodies that react specifically with AlHV–1 in species belonging to four subfamilies of the family *Bovidae*. They were members of the *Alcelaphinae*, which include red hartebeest (*Alcelaphus buselaphus*), topi (*Damaliscus korrigum*) and blesbok (*Damaliscus dorcas phillipsi*); the *Hippotraginae* such as the scimitar-horned oryx (*Oryx dammah*), gemsbok (*Oryx gazella*), waterbuck (*Kobus ellipsiprymnus*), Nile lechwe (*Kobus megaceros*), sable (*Hippotragus niger*) and roan (*Hippotragus equinus*); the *Caprinae*, which include all the sheep and goat species; and the *Ovibovinae* such as the musk ox (*Ovibos moschatus*)[16,28,55].

Related gammaherpesviruses have been isolated from the topi[45] and red hartebeest[54] and designated *alcelaphine herpesvirus* 2 (AlHV–2) and in roan antelope, which has been designated *hippotragine herpesvirus* 1 (HipHV–1)[51]. Experimentally AlHV–2 induced MCF in cattle and rabbits and HipHV–1 induced MCF in rabbits[51,55]. Their role in the epidemiology of MCF is, however, regarded as negligible[56]. These viruses are genetically distinct from AlHV–1 and OvHV–2. Experimentally AlHV–1 has been shown to induce MCF in cattle, guinea pigs, rabbits, hamsters and rats; while OvHV–2 induced MCF in species of cattle, deer, rabbits and hamsters[13,18,23,52]. Two new gammaherpesviruses have been recognized in springbok and were named *springbok herpesvirus* 1 (SpHV–1) and 2 (SpHV–2); as well as one in impala, *impala*

herpesvirus 1 (ImHV–1)[47]. The pathogenicity of these viruses in other animals is, however, not known.

1.7 DIAGNOSTIC TESTS

Currently the diagnosis of MCF affected animals in South Africa is based on the use of polymerase chain reaction (PCR) assays[12]. Histopathology remains, however, the most definitive diagnostic procedure. Additional diagnostic tests used for sero-surveillance are based on the detection of antibodies against AlHV–1. Serological diagnostic tests such as the neutralisation and complement fixation test[59,63]; enzyme-linked immunosorbent assay[28] (ELISA); and immunofluorescence and immunoperoxidase tests[58] have been described, but none of these tests are convenient for routine field testing for antibodies reactive to MCF-causing viruses since they are either cumbersome, expensive or not acceptably sensitive[40].

A competitive inhibition ELISA (CI-ELISA) was developed and detects MCF antibodies in ruminant sera or plasma[29]. Malignant catarrhal fever antibodies inhibit the binding of labelled MCF-specific monoclonal antibody 15-A to viral antigen coated plastic wells. Monoclonal antibody 15-A targets an epitope on the viral glycoprotein complex conserved among MCF virus isolates[26]. Binding of the labelled monoclonal antibody conjugate is detected by adding enzyme substrate and quantified according to the colour product development. Strong colour development indicates little or no inhibition of the binding of labelled monoclonal antibody to the viral antigen coated wells. This implies the absence of MCF antibody in the sample sera. Inhibition of binding of labelled monoclonal antibody results in weak colour development and indicates the presence of MCF antibodies in the sample sera[29].

The CI-ELISA is dependent upon the presence of a single epitope (15-A) common to the MCF viruses. It is expensive and therefore not often used in developing countries. The specificity was proven to be high (91 – 100%) but not so sensitive (56 – 87%)[17].

Alcelaphine herpesvirus type 1 can be isolated and propagated *in vitro* with relative ease. Virus is excreted by the natural host, the wildebeest, in a cell-free form for a brief period following infection and can be isolated from nasal swabs and blood leukocytes during that period[33]. Viral isolation from clinically affected animals can be achieved from cell suspensions of either peripheral blood leukocytes, lymph nodes or other affected tissues[33]. The primary structure of the genome of the virus has been determined[8,14] and PCR assays and hybridisation are used to detect the virus in tissues of animals infected naturally[35,36,38], as well as experimentally[37,47].

In contrast, it has not been possible to isolate OvHV–2 by conventional methods. It has been possible, however, to generate lymphoblastoid cell lines from MCF affected animals that have been shown to contain DNA sequences which cross-hybridise with some clones of the unique region of the AlHV–1 genome[9,10,53]. Clones of viral DNA have been derived from one such cell line and regions specific to OvHV–2 have thus been sequenced. This forms the basis for the PCR detection of the virus in clinical cases and in carrier sheep[6,7]. This PCR assay has successfully been used to amplify material originating from the United States of America (USA), United Kingdom (UK) and Indonesia[70]. It has been shown that OvHV–2 infect all domestic sheep. This has been confirmed by using the PCR test as well as serological assays, which rely on detecting antibodies cross-reacting with AlHV–1[19,49].

A single-tube nested PCR was developed by Dungu *et al*[12] for the detection of OvHV–2 in clinically affected animals as well as carrier sheep. This assay is very sensitive and specific for OvHV–2 and can be used to distinguish between sheep-associated MCF and wildebeest-associated MCF[12].

A hydrolysis probe format was used in the paper by Traul *et al*[68]. This assay targeted the gene coding for a tegument protein, open reading frame (ORF) 3. In this study, the real-time PCR was compared to a nested conventional PCR that amplified ORF 50 that coded for a transactivator protein. According to the authors, the nested PCR was more sensitive (≈1 log) than the real-time PCR[68].

1.7.1. REAL-TIME PCR

Real-time PCRs are based on the detection and quantification of a fluorescent reporter signal during the PCR amplification process[57]. Fluorescence is measured during each cycle of amplification and the amount of emitted fluorescence is directly proportional to the amount of PCR product[57]. Different approaches to real-time PCR exists and it includes the use of fluorescent DNA binding dyes, such as SYBR Green dye and ethidium bromide, or sequence specific probes, such as hydrolysis and hybridisation probes, molecular beacons and scorpions[57]. The sequence specific probes are labelled with FRET (fluorescent resonance energy transfer) fluorophores[57]. The fluorescent DNA binding dyes, e.g. SYBR Green, are the least costly and very sensitive but will detect both specific and non-specific PCR products. The sequence specific assays are more expensive but will only produce fluorescent signal if a specific product is present in the reaction[57].

SYBR Green is a fluorescent nucleic acid binding dye that binds to the minor groove of double stranded DNA (dsDNA). SYBR Green fluoresces when it is unbound but fluorescence is greatly enhanced when it binds to dsDNA. Fluorescence emission is read at 530 nm. The PCR product (amplicon) is detected by measuring the increase in fluorescence caused by binding of SYBR Green to dsDNA. SYBR Green, however, does not discriminate between different dsDNA strands in reaction solution. Any primer artefacts, such as primer-dimers and non-specific reaction products, will contribute to an increased fluorescent signal measurement, resulting in an overestimation of PCR target sequence concentration. It is therefore necessary to generate a melting or "dissociation" curve to differentiate between specific and non-specific PCR products (Figure 1.2)[57]. Melting curve analysis is based on the melting temperature (T_m) of the amplified product, which is determined by the length of the amplicon and the percentage GC content. This is the temperature at which the strands of amplicon will dissociate. The fluorescent signal is continuously monitored and plotted against temperature. This forms the "dissociation" or melting curve (Figure 1.2)[57].

Primer-dimer products result from the extension of one primer, using the other primer as a template, even though no stable annealing of the primer to any segment of the target DNA sequence is possible. As such an extension occurs, it becomes a valid template for PCR amplification as it contains both primer-annealing sites. These primer-dimer sequences are shorter than the target PCR sequences, and can thus be detected using melting curve analysis. These products have lower melting temperatures than full-length amplicons. Primer-dimer formation is most prevalent in negative

control reaction mixtures without template DNA to anneal to or in reaction mixtures with low concentrations of template[57].

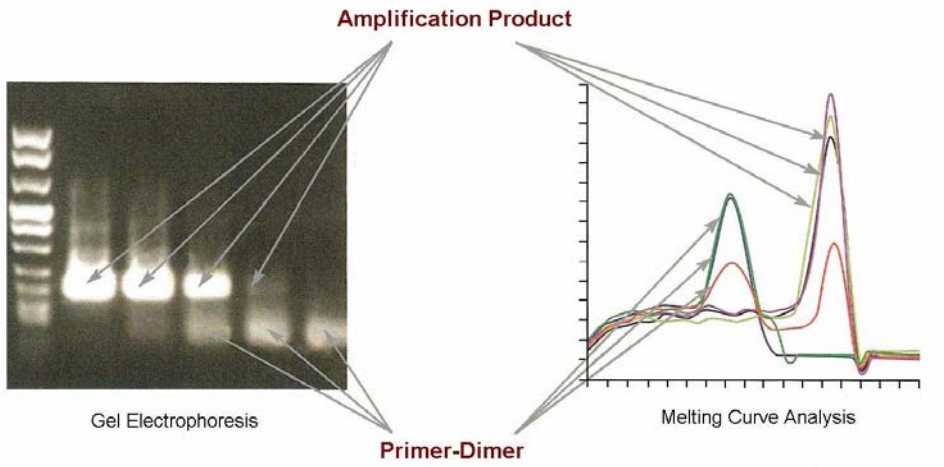

Figure 1.2: Visualization of amplified product and primer-dimers[57]

Real-time PCR is suitable for diagnostic applications, gene expression analysis, pathogen detection, bacterial and viral load determination, and allelic discrimination. It has been used successfully in research in detecting AIHV–1 DNA[68].

1.8 TRANSMISSION OF THE VIRUS

The virus probably spreads as an intense epizootic in the perinatal sheep lamb flock or wildebeest calves. However, there is evidence that a proportion of wildebeest foetuses become infected *in utero*[1,2]. These calves probably act as a source of infection to other calves and the virus subsequently establishes a latent infection with periodic episodes of recrudescence[64,65]. The infection therefore establishes in the population in all the individuals within the first months of life. The spread of the virus to other species is not

understood and one of the factors that might play a role is viral receptor density. The virus appears to be excreted especially via the ocular and nasal secretions from wildebeest calves[43] and sheep lambs. It is only the cell-free virus secreted mainly by the calves or lambs that is infective to susceptible species.

1.9 ECONOMIC EFFECTS

All blue wildebeest sera have antibody titres[48]. In Kenya it is the vast herds of blue wildebeest which calf especially during the migration that serve as a source of infection for cattle. This leads to conflict between wildlife managers and cattle owners. Losses of cattle due to MCF were calculated to be up to 7% annually in those areas in Africa where wildebeest and cattle intermingle[68]. This has significant economic implications for those farmers. In Zimbabwe and South Africa the same problems arise between game farm owners and neighbouring cattle farmers. Although the infection in cattle in the past was thought to be fatal, carrier animals have been identified in South Africa[36].

The economic effects of MCF were regarded as significant enough to include the disease in the list of controlled diseases in South Africa. Legislation to control MCF in South Africa was enacted in 1984 under the Animal Diseases Act and MCF was declared a controlled disease. Every outbreak and number of deaths had to be reported. Farmers were given authorisation up to 1987 to register their properties for keeping wildebeest, but thereafter registration was only possible if several strict requirements were complied with. Control methods included restrictions on the movement of wildebeest and were only allowed if a veterinary permit was issued for translocation. Animals were also removed from unregistered properties.

Game farmers and all related parties involved in eco-tourism and hunting formed a strong lobby to convince the Minister of Agriculture that even with all the control measures implemented to regulate the distribution and movement of wildebeest, MCF had not been significantly controlled. The appeal of these groups was also based on the importance of the wildebeest to game farming, hunting and the agricultural economy. It pointed out the discriminative and prescriptive nature of legislation regulating the free trade and movement of wildebeest. The importance of game farming as a form of land-use in the semi-arid savannah regions was accentuated. In addition, the occurrence of OvHV–2, carried by sheep made rational control strategies very difficult. The appeals yielded success and the control measures for MCF and restrictions on wildebeest movement were lifted in April 1993[56].

With the unrestricted movement of wildebeest, a simultaneous increase in MCF cases in cattle have been seen as result of the increased contact between black and blue wildebeest and domestic cattle at the wildlife-livestock interface. Many farmers opted to change from cattle to game farming due to the more lucrative nature of game farming, especially in the more arid regions, but also because of the increased occurrence of MCF in their cattle. This occurred as result of an increase in wildebeest populations on neighbouring farms. Between 1993 and 1999, the number of game farms in Limpopo Province has doubled. The number of fenced game farms is increasing annually by 3% (National Department of Agriculture of South Africa, 2000).

1.10 CONTROL MEASURES

Attempts to develop a vaccine have proven to be unsuccessful[39]. The only reliable control measure at present is to keep cattle separated from potential reservoir hosts such as wildebeest and sheep[68]. It is, however, regarded as necessary to separate wildebeest and cattle by at least a few hundred meters to prevent infection in cattle. At present a 1000 m corridor is regarded as the minimum distance of separation necessary to limit the risk of virus transmission to the absolute minimum (500 m would diminish the risk by 80%)[2]. It is also suggested that cattle be kept away from wildebeest during and for three months after the wildebeest calving season[43]. This is, however, not very practical and would be very difficult to implement by small-scale farmers bordering on conservation areas and game reserves. Traditional grazing land usually borders these conservation areas and it would be impossible not to utilise adjacent grazing areas in these restricted and often dry areas.

1.11 THE ROLE OF BLACK WILDEBEEST

South Africa is the only natural habitat of the black wildebeest. In other countries they are only found in zoological collections. The numbers of black wildebeest and its geographic distribution have increased dramatically during the last three decades. This is a result of the growth and expansion of the eco-tourism and wildlife industry in South Africa that includes controlled hunting and live capture and sale of animals. So much so that it's geographic distribution in South Africa is probably now greater than that of the blue wildebeest[2].

Up to now, blue wildebeest has been regarded as the most important carrier of AlHV–1. However, black wildebeest has been emerging as an important cause of MCF in recent years. A

comparison of black wildebeest-associated outbreaks between the years of 1981 – 1983, compared to 1988 – 1990, revealed that cases of MCF where only black wildebeest were involved have increased seven-fold[2]. This increase can be ascribed to the increase in the number of farms on which black wildebeest are kept.

No viral studies in the black wildebeest have been reported and the nature of the virus carried by black wildebeest is not known. Molecular techniques to amplify viral nucleic acids extracted from black wildebeest blood and tissue samples with primer sequences specific for AlHV–1 or OvHV–2, might therefore be unsuccessful.

Research on free-ranging black wildebeest can only be conducted in southern Africa. Their presence on game farms and in conservation areas in significant numbers provides an excellent opportunity to establish the gammaherpesvirus carrier status of black wildebeest. With the exception of one paper in a non-peer reviewed journal[2] and one published article by Mettam[34], publications that focus on the black wildebeest as a reservoir host for MCF are lacking.

1.12 THE AIMS OF THE RESEARCH PROJECT

The primary aims of the project were to:

1. **Isolate an MCF-causing virus from a black wildebeest calf in tissue culture.**
2. **Develop and standardise a molecular-based diagnostic test, specifically a real-time PCR assay, for detection of the MCF-causing gammaherpesvirus carried by black wildebeest.**
3. **Test specimens from black wildebeest representing a wide geographic area, for the presence of an MCF-causing virus or antibodies reactive to an MCF-causing virus.**
4. **Determine the carrier status of black wildebeest for an MCF-causing virus.**

Chapter 2

MATERIALS AND METHODS

2.1 STUDY AREA

Specimens were collected from several game farms and conservation areas across central South Africa to include a broad population of black wildebeest. Samples were collected from black wildebeest that were culled by the culling team of Free State Nature Conservation, as well as from animals caught by a professional capture team, Thaba Manzi Wildlife Services.

The culling operation is a standard part of Free State Nature Conservation's yearly population control of different species, in conjunction with live capture. The quota of live animals and carcasses sold annually by Free State Nature Conservation is put on auction before the start of the capture and hunting season in March. Some carcasses are allocated to welfare organisations, schools and retirement homes. The animals were culled on the following reserves: Tussen-die-Riviere (TDR), Gariep (GP), Maria Moroka (MM) and Sandveld (SAV) Nature Reserves (Figure 2.1).

Excess animals are removed from private game farms by means of live capture and translocation as a management tool to keep numbers down and as a source of income. The animals caught by the capture team were relocated to other game farms after the capture. The animals were caught on the farms "Schoondal" (TD), "Grootkoppe" (EE) and "Copperfield" (JF) that are situated near Potchefstroom in Northwest Province (Figure 2.1).

Figure 2.1: The different nature reserves and private game farms where black wildebeest were culled or captured and samples collected in central South Africa. (Courtesy of the Department of Environmental Affairs and Tourism, South Africa.)

2.2 STUDY POPULATION

Samples were obtained from 304 black wildebeest of different ages and gender, as well as 51 black wildebeest foetuses at different stages of gestation. The animals were selected according to what became available during different capture and culling operations.

Sera for serology (n=210) and EDTA-blood (n=212) and tissue samples (n=371) for conventional and real-time PCR testing were collected from the black wildebeest that were culled in the Free State nature reserves during 2005 (Table 2.1). Cornea and spleen samples from 51 foetuses were also collected during the culling

operations (Table 2.1). The animals were shot at night with long-range rifles fitted with silencers and immediately bled. Tissue samples were taken when the carcasses were skinned and eviscerated.

Blood samples in serum tubes (n=89) and EDTA-coated tubes (n=89) were also collected from live animals caught by Thaba Manzi Wildlife Services (Table 2.1). They were herded with a helicopter into a funnel-shaped net corral. As they were removed from the nets to be tranquillised and loaded, blood samples were taken from either the jugular or saphenous vein.

Table 2.1: Summary of the total number of samples collected.

Farm/Reserve	Serum	EDTA	Spleen	Cornea	Foetal spleen	Foetal cornea
Samples from black wildebeest culled						
TDR	43	43	34	42	9	9
JF	2	2	2	2	0	0
MM	58	58	22	58	8	8
GP	69	71	67	68	28	28
SAV	38	38	38	38	6	6
	210	**212**	**163**	**208**	**51**	**51**
Samples from black wildebeest captured live						
TD	27	27	0	0	0	0
EE	32	32	0	0	0	0
JF	30	30	0	0	0	0
	89	**89**	**0**	**0**	**0**	**0**
Total number of samples collected						
	299	**301**	**163**	**208**	**51**	**51**

Furthermore, a 10 ml heparinised blood sample was collected from a black wildebeest calf (WB5) that was born in the holding pens at Thaba Manzi Wildlife Services at one week of age. This sample was immediately taken to the virology laboratory at the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, and processed for viral isolation.

A young buffalo bull (BB) (*Syncerus caffer*) was housed with some older black wildebeest calves for six months in the holding pens of Thaba Manzi Wildlife Services. As buffaloes are known to be susceptible to MCF in captivity and under stressful conditions, blood samples were collected to determine if the animal seroconverted during this period of time.

These operations would have been carried out irrespective whether samples were taken or not by the culling and capture team who has 20 years of experience in their respective fields. Consent to obtain samples from carcasses and live animals were obtained from Free State Nature Conservation and Thaba Manzi Wildlife Services.

2.3 SPECIMEN COLLECTION, PREPARATION AND STORAGE

The date and conservation area involved were used to identify samples by using an abbreviation for each conservation area as well as 3 numbers allocated to each sample of which the first number referred to the reserve or farm and the second and third numbers referred to the animal's number.

For each animal the sex and the class of animal was noted. Animals were classed as adult (older than two years of age), sub-adult (one to two years of age) or juvenile (less than one year of age). The foetuses were measured in centimetres and in older foetuses the sex was also noted (Appendix I).

Sampling method for carcasses:

1. The carcasses were numbered numerically by using a tag with the number attached to the hock of the animal. The corresponding samples were marked accordingly. Two blood

samples were collected from each animal as soon as it was shot and the throat cut for the carcass to bleed out. Blood was collected in a 10 ml serum and 7.5 ml EDTA-coated Vacutainer™ tube (BD Vacutainer Systems, Preanalytical Solutions). EDTA containing blood samples were transferred to sterile 2.0 ml Cryovials® (Simport Plastics) and stored at –70°C. One cornea was removed using a sterile scalpel blade and a piece of the spleen removed with a sterile scalpel blade. These samples were stored frozen in sterile containers at –20°C.

2. If an adult cow was pregnant, the foetus was removed and an eye and the spleen removed and placed in sterile containers and stored at –20°C.

Sampling method for live animals:

1. As soon as the animals were captured in the net corral and handled to be injected with intravenous tranquillisers, two blood samples were collected from each animal by venipuncture of the jugular or saphenous vein using an 18G Vacutainer™ needle (BD Vacutainer Systems, Preanalytical Solutions). Blood was collected in a 10 ml serum and a 7.5 ml EDTA-coated Vacutainer™ tube (BD Vacutainer Systems, Preanalytical Solutions). EDTA containing blood samples were transferred to sterile 2.0 ml Cryovials® (Simport Plastics) and stored at –70°C.

2.4 SEROLOGY

The serum blood samples were centrifuged at 440 x g for 10 minutes and the serum removed and stored in sterile 2.0 ml Cryovials® (Simport Plastics) at –20°C.

A direct competitive inhibition ELISA kit (VMRD Inc©, USA) for detection of malignant catarrhal fever virus antibody in ruminant sera or plasma was used[29]. The kit consisted of an antigen-coated plate with 96 wells, a positive and negative control, antibody peroxidase conjugate, serum/conjugate diluting buffer and substrate solution ready to use. The preparation of the serum samples and the test procedure were done according to the manufacturer's instructions.

Binding of the labelled MCF-specific monoclonal antibody 15-A conjugate to viral antigen is detected by adding enzyme substrate and quantified according to the colour product development. Strong colour development indicates little or no inhibition of the binding of labelled monoclonal antibody to the viral antigen-coated wells. This implies the absence of MCF antibody in the sample sera. The presence of MCF antibodies in the sample sera will inhibit binding of labelled monoclonal antibody and result in weak colour development[29].

The plate was read with an ultra microplate reader, Bio-Tek Instruments©, Model EL808 and the results prepared by the software program KC Junior©. If the test sample caused more than 25% inhibition, it was noted as positive. The following formula was used to determine the percentage inhibition from the raw data:

100 – [SAMPLE O.D. (AVE) X 100 / MEAN NEG. CONTROL O.D.] = %INHIBITION

Ninety test samples could be done on each plate. Two hundred and seventy serum samples were tested in total, which consisted of 269 black wildebeest samples and the young buffalo bull's (BB) sample. The positive and negative controls used were supplied as standard controls with the kit.

2.5 VIRAL ISOLATION AND IDENTIFICATION

Blood was collected in a heparin-coated Vacutainer™ tube (BD Vacutainer Systems, Preanalytical Solutions) from black wildebeest calf no 5 (WB5) at one week of age and co-cultivated with secondary black wildebeest foetal kidney (BWFK) cells established in the laboratories of the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria from a black wildebeest foetus (2004).

Equal volumes (2.5 ml) of buffycoat cells from the heparin-coated tube was mixed with sterile phosphate buffered saline minus calcium and magnesium (PBS Minus) and layered onto 3 ml Histopaque® (Farmacia) in a 15 ml conical tube and centrifuged at 400 x g for 30 minutes at room temperature. The interface of this density-gradient centrifugation product, containing the peripheral blood mononuclear cells (PBMCs), was removed with an electronic pipette and placed in another sterile 15 ml conical tube. Two millilitres of sterile PBS Minus was added and the cells washed by means of centrifugation at 150 x g for 10 minutes at room temperature to get rid of the ficol-hypaque and PBS Minus. The PBS Minus was aspirated and the washing step repeated with fresh PBS Minus.

The resuspended cell pellet was added to BWFK cells within 5 hours of collection of the blood and placed in an incubator at 37°C. The separated peripheral blood mononuclear cells were added to five-day-old confluent secondary BWFK cells of the 8th passage. The cell culture media consisting of minimum essential medium (MEM) plus 5% foetal calf serum were changed after 48 hours. Following the development of typical cytopathic effects in the cell monolayer, the supernatant and cell debris was examined for the presence of viral particles by means of electron microscopy. The DNA from this viral

isolate was extracted following the same extraction method as the field samples, sequenced and used as a positive control (Refer to 2.6 Molecular techniques).

The virus isolated was sent to the Electron Microscopy Unit, Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria, for confirmation as a herpesvirus. The tissue culture was spun down at 15 000 x g to form a pellet containing the virus particles and the supernatant disposed of. The pellet was resuspended by adding a drop of water and mixed with a drop of 3% phosphotungstic acid. The formvar carbon coated grid was placed on the sample and studied through the Philips CM10 transmission electron microscope for identification.

2.6 MOLECULAR TECHNIQUES

2.6.1 CONTROLS

The Biotechnology Section of the Onderstepoort Veterinary Institute in Pretoria provided an EDTA-containing blood sample of a bovine (Bovine 4408) that was confirmed to have died of wildebeest associated MCF as result of nearby grazing blue wildebeest. The second positive control was the viral isolate from the black wildebeest calf, WB5. The viral isolate obtained from WB5 was used as a positive control for the real-time PCR assays, while Bovine 4408 was used as a positive control for both conventional and real-time PCR.

An OvHV–2 positive sheep sample of Norwegian origin, tested by the laboratory of Veterinary Tropical Diseases, Faculty of Veterinary Science, Onderstepoort, University of Pretoria, was used as a negative control in certain conventional PCR assays. Double distilled sterile water was used as the standard negative control in all assays.

2.6.2 DNA EXTRACTION

DNA was extracted from 200 µl of whole blood or two grams of tissue using the QIAamp® DNA Mini Kit (QIAGEN©, Southern Cross Biotechnologies) according to the manufacturer's instructions. Extracted DNA was eluted in 100 µl elution buffer and stored at –20°C.

2.6.3 CONVENTIONAL PCR

Amplification of viral DNA was initially attempted by means of a conventional PCR assay to detect viral nucleic acid in the samples. Several different PCR assays previously described in the literature were evaluated for their ability to detect AlHV–1 viral DNA in black wildebeest samples using the published conditions (Table 2.2). These were optimised, if necessary, to obtain the best possible results. The optimised conditions are described below.

Table 2.2: A summary of the PCR assays evaluated, references and melting temperatures (T_m)

Reference	Primer name	Primers	T_m	
			Min	Max
Murphy *et al*[42]	A	5'–TACATGTCATTTAAGACACCCACGCACCA–3'		
	B	5'–CTGGTGCAGGATGACCACAAATTTTACTATC–3'		
VanDevanter *et al*[69]	DFA	5'–GAYTTYGCNAGYYTNTAYCC–3'	52.20	66.55
	ILK	5'–TCCTGGACAAGCAGCARNYSGCNMTNAA–3'	64.61	73.40
	KG1	5'–GTCTTGCTCACCAGNTCNACNCCYTT–3'	64.59	70.90
	TGV	5'–TGTAACTCGGTGTAYGGNTTYACNGGNGT–3'	64.62	71.69
	IYG	5'–CACAGAGTCCGTRTCNCCRTADAT–3'	61.15	67.98
Flach *et al*[16]	Pol1	5'–GGCCTCACTAACTCTATGCTACTCCAC–3'	62.86	67.98
	Pol2	5'–ATTAGTCCACAAACTGTTTTGT–3'	52.80	54.76
	AlHV–Pol	5'–CCAAAATGAAGACCATCTTA–3'	54.25	54.25
	OvHV–Pol	5'–AAAAACTCAGGGCCATTCTG–3'	58.35	58.35

Reference	Primer name	Primers	T_m	
			Min	Min
Li *et al*[25]	C500–1	5'–TACGGGTGCCCTGACATTTCATCTCTTTTG–3'	67.37	67.37
	C500–2	5'–ATAACTGGTTGATGTGGCAGATGCATCTAT–3'	64.63	64.63
	C500–3	5'–TCTGGCCCGTGCTGCAGCAAGACTCTCAG–3'	73.11	73.11
	C500–4	5'–TATAGTAGAATCCCGTCTGAGTGGTAGCTG–3'	67.37	67.37

T_m	=	melting temperature	Y	=	T or C (Two-fold degenerate)
A	=	adenine	N	=	G, A, T or C (Four-fold degenerate)
T	=	thymine	M	=	A or C (Two-fold degenerate)
C	=	cytosine	R	=	A or G (Two-fold degenerate)
G	=	guanine	S	=	G or C (Two-fold degenerate)

- **PCR ASSAY DESCRIBED BY MURPHY *ET AL*[42]**

These primers amplify a 202 bp region of the genome of AlHV–1 that stretches over genes 22 and 23 (Figure 2.2). Murphy *et al*[42] used Southern blot hybridisation to confirm the identity of the DNA bands after amplification. It was not done in this study.

The reaction mixture consisted of 12.5 µl of Platinum® Quantitative PCR SuperMix-UDG (Invitrogen™ Life Technologies, The Scientific Group), 10 ρmol of each primer (A and B) (Inqaba Biotech, SA) (Table 2.2), 2.5 µl DNA, made up with sterile double distilled water to a final volume of 25 µl. DNA extracted from 32 black wildebeest samples were tested. DNA from Bovine 4408 was used as the positive control and the OvHV–2 positive sheep sample and distilled water as negative controls.

Amplification was performed in an automated thermocycler (Perkin Elmer, Applied Biosystems). An initial pre-cycle incubation at 42°C for 2 minutes was followed by a denaturation step at 94°C for 10

minutes. This was followed by 35 cycles, each cycle consisting of denaturation at 94°C for 30 seconds; annealing at 65°C for 60 seconds; and extension at 72°C for 30 seconds. Final extension was at 72°C for 60 seconds before cooling down to 4°C. In order to optimise the PCR conditions, the annealing temperature was subsequently lowered to 60°C and 55°C, respectively.

For further optimisation, the effect of increased magnesium chloride ($MgCl_2$) concentrations (3 mM, 6 mM and 12 mM) and varying DNA input concentrations, was also investigated. DNA input was first increased from 25 ng to 50 ng, 75 ng and 100 ng. Thereafter the DNA input of 25 ng was decreased by a series of dilutions (1 in 10; 1 in 50; 1 in 100; 1 in 150; 1 in 300; and 1 in 500). DNA of Bovine 4408 and sample 572 were used as test samples for optimisation.

The amplified DNA fragments and molecular size markers (O'Gene Ruler™ 100 bp DNA Ladder, Fermentas Life Sciences) were loaded on a 2% agarose gel and subjected to electrophoresis. The gel was stained with ethidium bromide and the gel-separated bands visualised and photographed under ultraviolet light (UV-light).

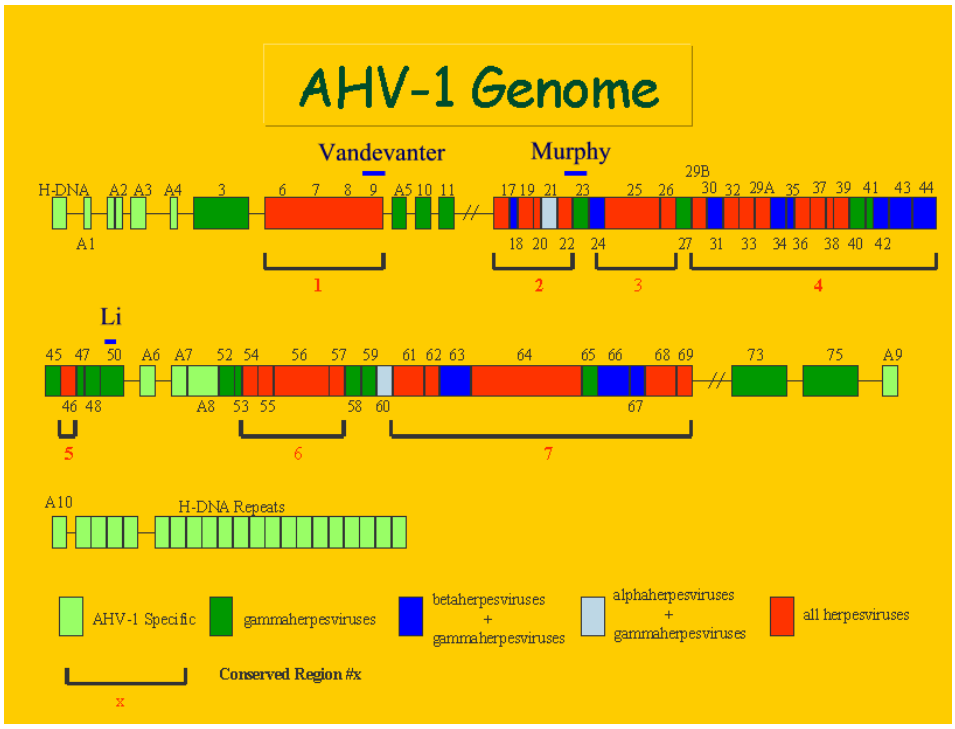

Figure 2.2: A diagrammatic description of the AlHV–1 genome (Courtesy Dr J C DeMartini, Colorado State University). Primer positions are indicated above for the PCR assays as described by Murphy *et al*[42], VanDevanter *et al*[69] and Li *et al*[25].

- **PCR ASSAY DESCRIBED BY VANDEVANTER *ET AL*[69]**

This assay uses degenerate primers in a nested format, targeting a highly conserved area within the herpesviral DNA-directed DNA polymerase gene, gene 9 (Figure 2.2). The assay amplifies a short region between 215 and 315 bp. In this PCR, DNA from Bovine 4408 was used as the positive control. An equine herpesvirus positive sample (V22), used as the positive control in routine diagnostic tests in the laboratory of Veterinary Tropical Diseases, Onderstepoort, was also included. Seven black wildebeest samples were tested.

The reaction mixture for the first step consisted of 12.5 µl of ReadyMix™ with $MgCl_2$ (Sigma-Aldrich, SA), 20 ρmol of each primer

(DFA, ILK and KG1) (Inqaba Biotech, SA) (Table 2.2) and 2.5 µl DNA, made up with double distilled sterile water to a total volume of 25 µl. The reaction mixture for the nested step contained 12.5 µl of ReadyMix™ with $MgCl_2$ (Sigma-Aldrich, SA), 20 ρmol of primer TGV and IYG (Inqaba Biotech, SA) (Table 2.2), respectively, and 2.5 µl DNA, made up to a total volume of 25 µl with double distilled sterile water.

The reaction mixtures for both the first and second steps in the nested PCR were subjected to the same cycling conditions, using an automated thermocycler (Perkin Elmer, Applied Biosystems). Initial denaturation was performed at 94°C for 12 minutes. The mixtures were then cycled 10 times at 94°C for 30 seconds; 68°C for 30 seconds with a decrease in temperature by 2°C with every cycle; and 72°C for 90 seconds. This was followed by 40 cycles at 94°C for 30 seconds; 48°C for 30 seconds; and extension at 72°C for 90 seconds. Final extension was at 72°C for 7 minutes and then held at 4°C.

The amplified DNA fragments and were visualised on a 2% agarose gel as described before.

- **PCR ASSAY DESCRIBED BY FLACH *ET AL*[16]**

A nested PCR was employed using primers described by Flach *et al*[16]. For the first amplification non-specific herpesvirus primers, Pol1 and Pol2 (Table 2.2) were used. For the second amplification, Pol2 and AlHV–Pol or OvHV–Pol primers (Table 2.2) were used and these primers target an area specific and unique to AlHV–1 and OvHV–2, respectively. This is used to distinguish between AlHV–1 and OvHV–2 positive samples. The size of the first amplification product was expected to be 386 bp and that of the second

amplification product 172 bp. Four black wildebeest samples were tested with these primers, as well as the positive controls, Bovine 4408 and the OvHV–2 positive ovine sample.

The reaction mixture for the first amplification step consisted of 12.5 µl of Platinum® Quantitative PCR SuperMix-UDG (Invitrogen™ Life Technologies, The Scientific Group), 10 ρmol of each primer, Pol1 and Pol2 (Inqaba Biotech, SA) (Table 2.2), 2 µl DNA and made up with sterile double distilled water to 20 µl in total. The reaction mixture for the nested amplification step consisted of 12.5 µl of ReadyMix™ with $MgCl_2$ (Sigma-Aldrich, SA), 10 ρmol of primer Pol2 (Inqaba Biotech, SA) (Table 2.2) and primer AlHV–Pol or OvHV–Pol (Inqaba Biotech, SA) (Table 2.2), respectively, 0.5 µl of amplified DNA from the first PCR and made up with double distilled water to a total volume of 20 µl.

Amplification took place in an automated thermocycler (Perkin Elmer, Applied Biosystems) with a pre-cycle incubation at 42°C for 2 minutes to deactivate the UDG and initial denaturation at 94°C for 10 minutes. Twenty-five cycles followed, each consisting of 30 seconds of denaturation at 94°C; 60 seconds of annealing at 45°C; and 60 seconds of extension at 72°C. Final extension was at 72°C for 7 minutes.

The amplified DNA fragments were visualised as described before.

- **PRIMERS AS DESCRIBED BY LI *ET AL*[25]**

A nested PCR was also tested using the primers described by Li *et al* (Table 2.2)[25]. These primers target a region in open reading frame (ORF) 50 (Figure 2.2) of the AlHV–1 genome[25] that codes for a transactivator protein which is associated with virulence in rabbits[18].

The outer primers amplify a fragment of 405 bp and this fragment is found from position 73917 to 74321 on the AlHV–1 genome. The inner primers amplify a fragment of 274 bp and is found from position 73986 to 74259[25]. The complete L-DNA sequence is available on GenBank (www.ncbi.nlm.nih.gov) and the accession number is AF005370[14]. Four black wildebeest samples, the positive control (Bovine 4408) and the negative control (OvHV–2 positive ovine sample) were tested.

The reaction mixture for the first amplification step consisted of 12.5 µl of Platinum® Quantitative PCR SuperMix-UDG (Invitrogen™ Life Technologies, The Scientific Group), 10 ρmol of primers' C500–1 and C500–2 (Inqaba Biotech, SA) (Table 2.2), respectively, and 2 µl DNA, made up to a total volume of 20 µl using sterile double distilled water. The reaction mixture for the nested amplification step consisted of 12.5 µl of ReadyMix™ with $MgCl_2$ (Sigma-Aldrich, SA), 10 ρmol of primers' C500–3 and C500–4 (Inqaba Biotech, SA) (Table 2.2) respectively, 0.5 µl amplified DNA from the first PCR step, made up to a total volume of 20 µl using sterile double distilled water.

Both the first and second amplification steps followed the same thermal cycling conditions using an automated thermocycler (Perkin Elmer, Applied Biosystems). Pre-cycle incubation was performed at 42°C for 2 minutes to deactivate the UDG, followed by a denaturation step at 94°C for 10 minutes. Thereafter 35 cycles followed, each cycle consisting of 30 seconds of denaturation at 94°C; 60 seconds of annealing at 55°C; and 60 seconds of extension at 72 °C. Final extension was at 72°C for 10 minutes.

The amplified DNA fragments were visualised as described before.

2.6.4 REAL-TIME PCR

A SYBR Green real-time PCR assay was developed using the nested primers C500–3 and C500–4 as described by Li *et al* (Table 2.2)[25]. These primers amplify a 274 bp region of the gene that codes for a transactivator protein, ORF 50 (Figure 2.2)[25].

All the black wildebeest samples were tested using this assay. Bovine 4408 and WB5 were used as the positive controls and double distilled sterile water as the negative control.

The amplification mixture consisted of 2 µl LightCycler® Faststart DNA Master SYBR Green I (Roche© Applied Science), 3 mM $MgCl_2$ (Roche© Applied Science), 10 ρmol of each primer (Inqaba Biotech, SA) (Table 2.2) and 2.5 µl extracted DNA made up with sterile double distilled water to a final volume of 20 µl.

Temperature cycling was performed in a LightCycler v2.0 (Roche©) and the real-time PCR results analysed by the Roche© LightCycler Software 4.0. An initial activation step at 95°C for 10 minutes was followed by 45 cycles of denaturation at 95°C with a 10 second hold; primer annealing at 65°C with a 10 second hold; and product extension at 72°C with a 20 second hold. Following amplification, a melting curve analysis was performed by heating the samples from 65°C to 95°C with a heating rate of 0.1°C per second. Fluorescence values were continuously measured at 530 nm.

For verification, some amplification products were visualised on a 2% agarose gel as described before. To confirm real-time PCR results, amplicons of the positive controls, WB5 and Bovine 4408, were subjected to sequence analysis.

2.7 SEQUENCE ANALYSIS

Real-time PCR amplicons of controls Bovine 4408 and WB5, which were used for the development and optimisation of the real-time PCR assay, were sequenced to confirm that the amplicons obtained were indeed the same as or closely related to AlHV–1. Real-time PCR products obtained from Bovine 4408 and calf WB5 were purified using the QIAquick® PCR Purification Kit (QIAGEN©, Southern Cross Biotechnologies) according to the manufacturer's instructions. The purified products were amplified using 1.0 µl AB1 Terminator Ready Reaction Mix (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems); 0.5 µl Buffer (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems); 3.2 ρmol of each primer C500–3 and C500–4 (Inqaba Biotech, SA) (Table 2.2), respectively; and 20 ng purified DNA product.

Cycling conditions, using an automated thermocycler (Perkin Elmer, Applied Biosystems), consisted of pre-cycle incubation at 96°C for 1 minute. Thereafter 25 cycles followed, each cycle consisting of 10 seconds of denaturation at 96°C; 5 seconds of annealing at 50°C; and 4 minutes of extension at 60°C. Final extension was at 72°C for 10 minutes.

After cycling, the amplified products were purified by incubating with 20 µl 96% ethanol for 15 minutes at room temperature in the dark. The samples were then centrifuged for 15 minutes at 10 000 x g, the ethanol removed and 80 µl 70% ethanol added. The samples were centrifuged for another 10 minutes at 10 000 x g and the ethanol removed. The samples were then dried in a heating block at 60°C until all drops of ethanol evaporated. The samples were sent to the Genetics Laboratory, Equine Research Centre, Faculty of Veterinary Science, University of Pretoria, to be sequenced. The samples were

sequenced using a 3130x1 Genetic Analyser and software AB Sequencing Analysis Software V5.2. Sequence data was edited to a total length of 268 bp using GAP4 of the Staden Package (Version 1.6.0 for Windows). Assembled sequences were aligned with published sequences of related viruses using ClustalX (Version 1.81 for Windows). A phylogenetic tree was constructed using the neighbor-joining method[60] in combination with the bootstrap method[15]. Using the BLASTn algorithm, a BLAST search was also performed to find any related DNA sequences using the PUBMED website (www.ncbi.nlm.nih.gov/BLAST/).

Chapter 3

RESULTS

3.1 SEROLOGICAL RESULTS

The black wildebeest samples (n=269) tested with the CI-ELISA, all yielded antibody reactive to the MCF group of viruses. The young buffalo bull (BB) that was included as an extra control, tested negative. Almost all of the samples, 97.3% (n=262), showed inhibition above 70% with one sample showing only 29% inhibition, one sample 46.5%, two samples between 50% and 59% and three samples between 60% and 69% inhibition (APPENDIX II).

3.2 VIRAL ISOLATION AND IDENTIFICATION

Cytopathic effects compatible with herpesvirus replication were visible 18 days after inoculation of cell cultures. The virus was identified by means of electron microscopy and an indirect fluorescent antibody (IFA) test using known positive polyclonal bovine serum. Viral particles were roughly 100 nm in diameter, enveloped and icosahedral in shape (Figure 3.1).

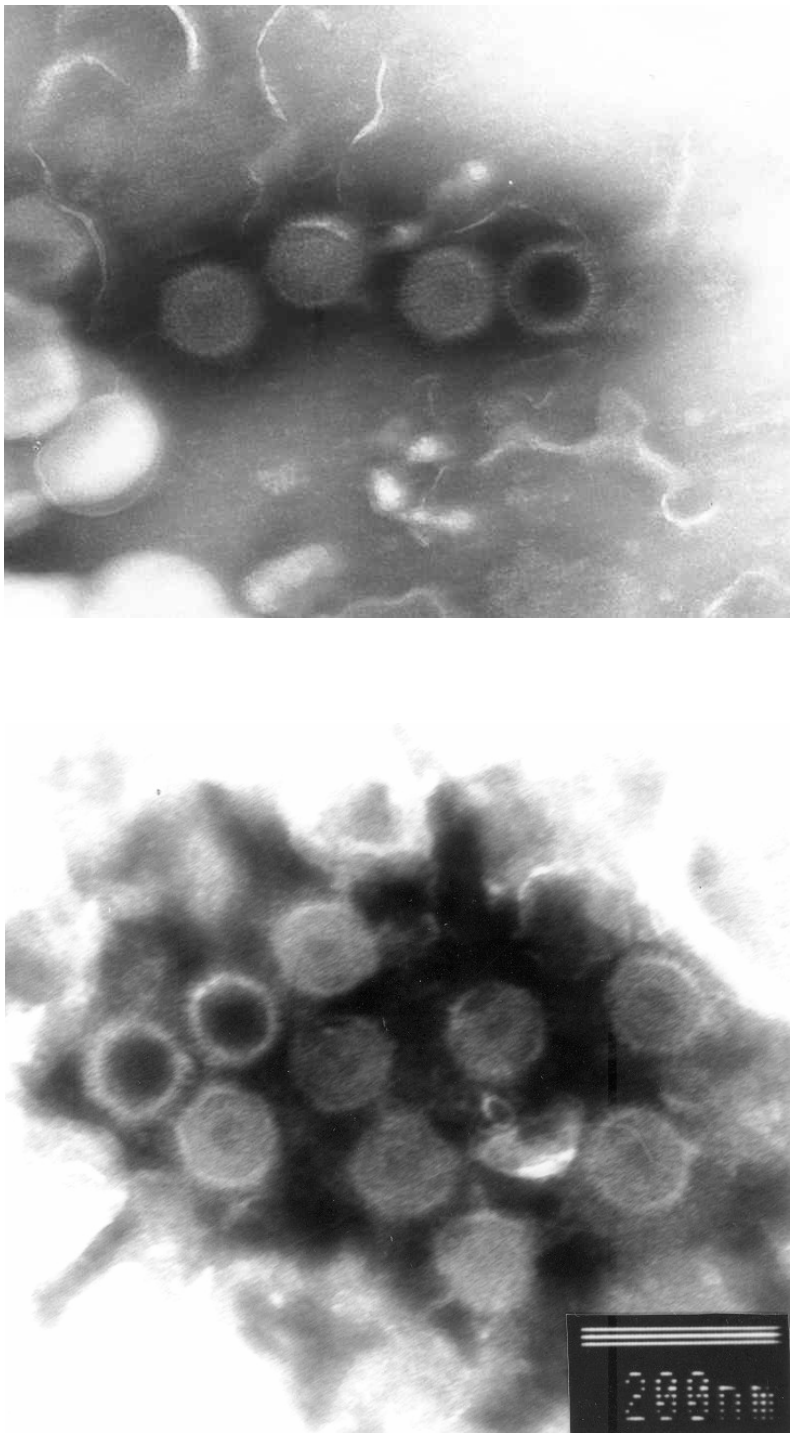

Figure 3.1: Electron micrographs of the virus isolated from the black wildebeest calf number 5 (WB5).

3.3 CONVENTIONAL PCR

Different PCR assays were evaluated for their ability to detect AlHV–1 viral DNA in black wildebeest samples. It was expected that the virus be present in very low numbers in adult animals.

- **PCR ASSAY DESCRIBED BY MURPHY ET AL[42]**

For the evaluation of the PCR assay previously described by Murphy *et al*[42], DNA extracted from 28 black wildebeest blood samples, one spleen sample and one cornea sample, as well as two foetal samples were tested using the conditions as described by the authors. Bovine 4408 was included as the positive control. DNA from Bovine 4408 yielded an amplified PCR product of the correct size (202 bp). None of the black wildebeest samples yielded PCR amplicons. The ovine negative control (OvHV–2 positive ovine sample) tested negative as expected. In the case of the foetal samples, only smearing was visualised on the gel under UV-light.

In order to optimise the PCR conditions, sample 572 and Bovine 4408 were used to evaluate the effect of different annealing temperatures (55°C and 60°C) (Figure 3.2). The higher annealing temperature of 60°C provided better amplification of Bovine 4408 DNA with a more visible product.

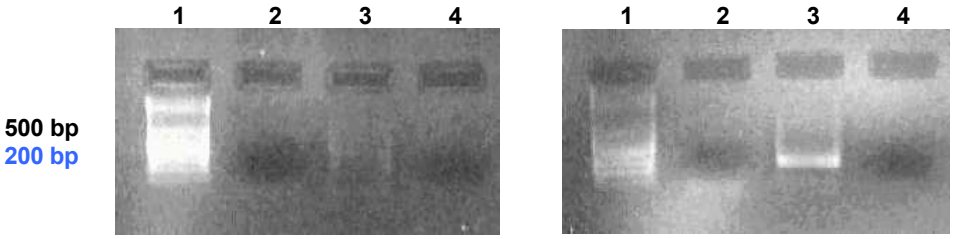

Figure 3.2: Agarose gel electrophoresis of ethidium bromide stained PCR products amplified using different annealing temperatures. (a) 55°C (b) 60°C. Lane 1 = DNA ladder. Lane 2 = sample 572. Lane 3 = Bovine 4408. Lane 4 = negative control (water).

The effect of different $MgCl_2$ input levels were also investigated to further optimise the PCR assay. Results showed no significant difference in the amplicons obtained (Figure 3.3).

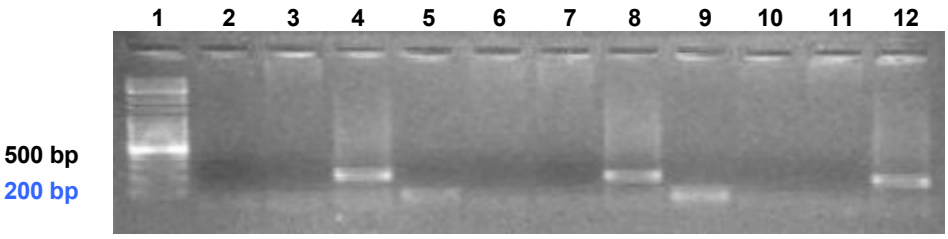

Figure 3.3: Agarose gel electrophoresis of ethidium bromide stained PCR products amplified using different $MgCl_2$ input concentrations. Lane 1 = DNA ladder. Lane 2 to 5 = 3 mM $MgCl_2$ added. Lane 6 to 9 = 6 mM $MgCl_2$ added. Lane 10 to 12 = 12 mM $MgCl_2$ added. Lane 2, 6 and 10 = Bovine 4408 (Extraction 1). Lane 3, 7 and 11 = Bovine 4408 (Extraction 2). Lane 4, 8 and 12 = Bovine 4408 (Extraction 3). Lane 5 and 9 = negative control water. (Extraction 1, 2 and 3 are different extractions from the same blood sample of Bovine 4408)

The effect of input DNA concentration was also investigated using sample 572 and Bovine 4408. The DNA input was first increased from 25 ng to 50 ng, 75 ng and 100 ng extracted DNA. Five different extractions of Bovine 4408 were used with each input. None of the increases in DNA input improved the amplification process. Most of the samples only showed smearing when visualised on the agarose gel under UV-light (Figure 3.4).

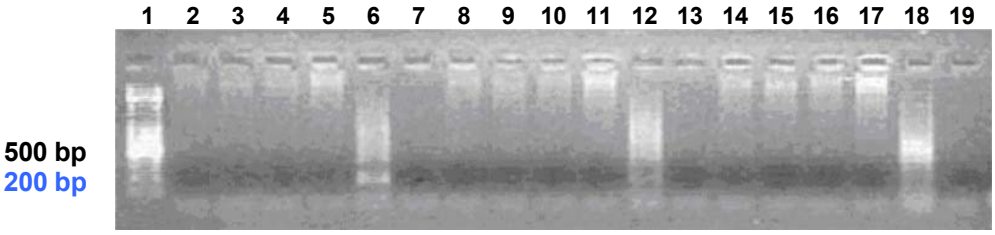

Figure 3.4: Agarose gel electrophoresis of ethidium bromide stained PCR products amplified from different extractions of Bovine 4408 and the negative control (water). Lane 1 = DNA ladder. Lane 2 to 7 = DNA input 50 ng. Lane 8 to 13 = DNA input 70 ng. Lane 14 to 19 = DNA input 100 ng. Lane 7, 13 and 19 = negative control (water)

As a result of the smearing shown after the increase in DNA input concentration, 25 ng DNA of sample 572 and Bovine 4408 was diluted to a 1 in10; 1 in 50; 1 in 100; 1 in 150; 1 in 300; and 1 in 500 dilution and subjected to PCR. Smearing was eliminated and amplicons of the correct size (202 bp) of only the undiluted and 1 in 10 dilution of Bovine 4408 DNA was obtained (Figure 3.5).

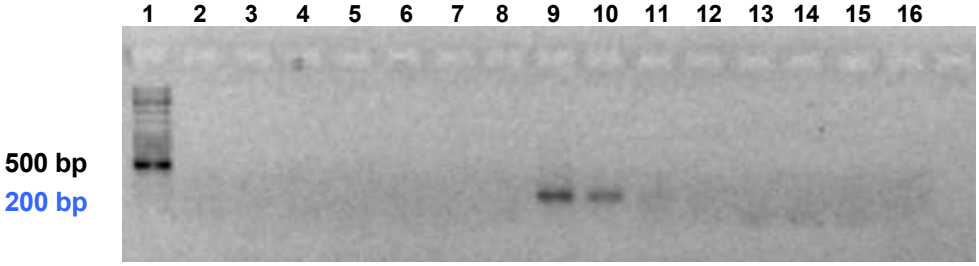

Figure 3.5: Agarose gel electrophoresis of ethidium bromide stained PCR products amplified from dilutions of sample 572 and Bovine 4408 with primers as described by Murphy *et* al[42]. Lane 1 = DNA ladder. Lane 2 to 8 = sample 572. Lane 9 to 15 = Bovine 4408. Lane 16 = negative control (water). Each sample was diluted to a 1 in 10, 1 in 50, 1 in 100, 1 in 150, 1 in 300, and 1 in 500 dilution. Lane 9 = Bovine 4408 undiluted (25 ng DNA). Lane 10 = Bovine 4408 in a 1 in 10 dilution.

The only aspect improving the PCR results was the decrease in the annealing temperature to 60°C. The best PCR conditions were

chosen from the above, i.e. annealing temperature = 60°C, $MgCl_2$ = 2.5 mM and a DNA input of 25 ng. To evaluate the optimised conditions, 32 spleen and cornea samples were tested. Only two samples (No. 4–1 (spleen) and no. 14–2 (cornea)) tested positive together with the positive control sample, Bovine 4408. Most of the other samples showed smearing on the agarose gel. To confirm that a too high DNA input was not the reason for the smearing, the DNA samples were diluted (1 in 10) and the PCR repeated. Although less smearing occurred, only sample number 14–2 produced an amplicon (Data not shown).

- **PCR ASSAY DESCRIBED BY VANDEVANTER *ET AL*[69]**

Seven black wildebeest samples as well as the positive control, Bovine 4408, tested negative on this PCR. The equine herpesvirus positive control (V22) gave an amplicon of between 215 and 315 bp after the nested step of the PCR (Figure 3.6). Two bands are visible for sample V22 after the nested step. These represent the amplified PCR products of V22 for both the first and nested step of the PCR assay.

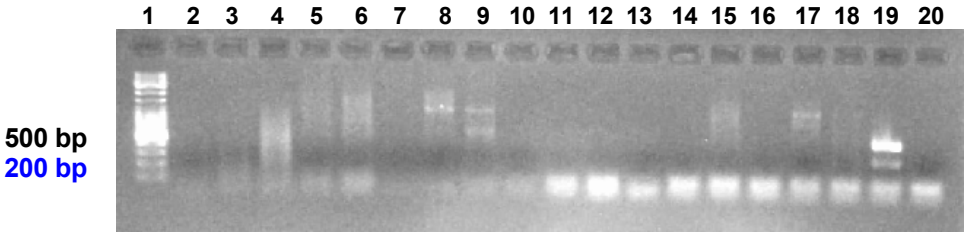

Figure 3.6: Agarose gel electrophoresis of ethidium bromide stained PCR products amplified from 7 black wildebeest samples, Bovine 4408 and equine sample, V22. Lane 1 = DNA ladder. Lane 2 to 10 = amplicons from the first amplification step. Lane 11 to 20 = amplicons from the nested step. Lane 9 and 18 = Bovine 4408. Lane 10 and 19 = equine herpesvirus control V22. Lane 20 = negative control (water).

- PRIMERS TESTED DESCRIBED BY FLACH ET AL[16]

Four black wildebeest samples were tested together with the positive control Bovine 4408 and two negative controls, an OvHV–2 positive sheep sample and water. All of the samples tested negative (data not shown). Some smearing was seen from sample 13–1 after the first amplification step but no amplicons could be visualised after the nested step.

- PRIMERS TESTED DESCRIBED BY LI ET AL[25]

Two black wildebeest samples were tested together with the positive control Bovine 4408 and two negative controls, an OvHV–2 positive sheep sample and water. Bovine 4408 yielded a 274 bp band only faintly visible on the agarose gel after the nested step (Figure 3.7).

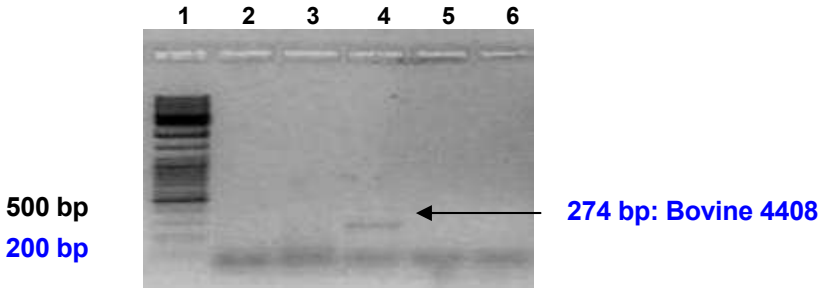

Figure 3.7: Agarose gel electrophoresis of ethidium bromide stained PCR products amplified with primers described by Li *et al*[25] after the nested step. Lane 1 = DNA ladder. Lane 2 and 3 = black wildebeest samples. Lane 4 = Bovine 4408. Lane 5 = negative control (OvHV–2). Lane 6 = negative control (water).

3.4 REAL-TIME PCR RESULTS

The positive controls, Bovine 4408 and WB5, were used to evaluate the newly developed SYBR Green real-time PCR assay. A melting curve analysis of the PCR products was performed to verify that the fluorescent signal obtained in the real-time PCR originated from specific PCR products and not from artefacts like primer-dimers (Figure 3.8, Figure 3.9). The average melting temperature (T_m) of

the amplified control, Bovine 4408 was 88.74°C (± 0.38°C) and control WB5 89.45°C (± 0.37°C). The negative water control had an average T_m of 85.03°C (± 0.56°C) (Appendix III). The latter is not unusual when SYBR Green is used and is caused by primer-dimer formation. This phenomenon was confirmed by visualising the products on an agarose gel (Figure 3.10).

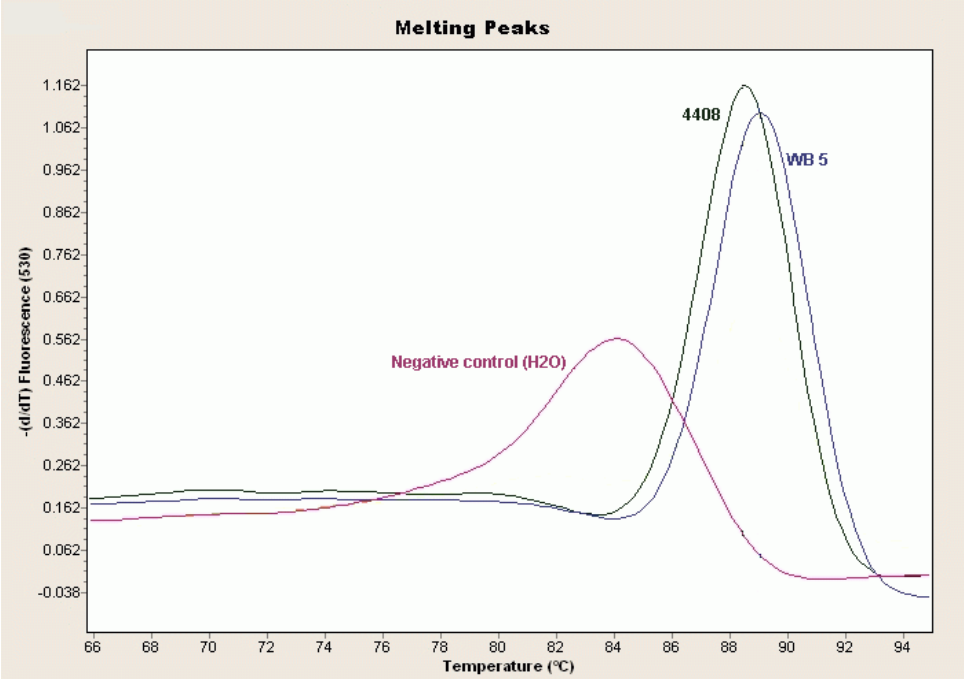

Figure 3.8: The melting curve for the two positive controls, Bovine 4408 and WB5, as well as the negative control, water. The peak of the negative control is as a result of primer-dimer formation.

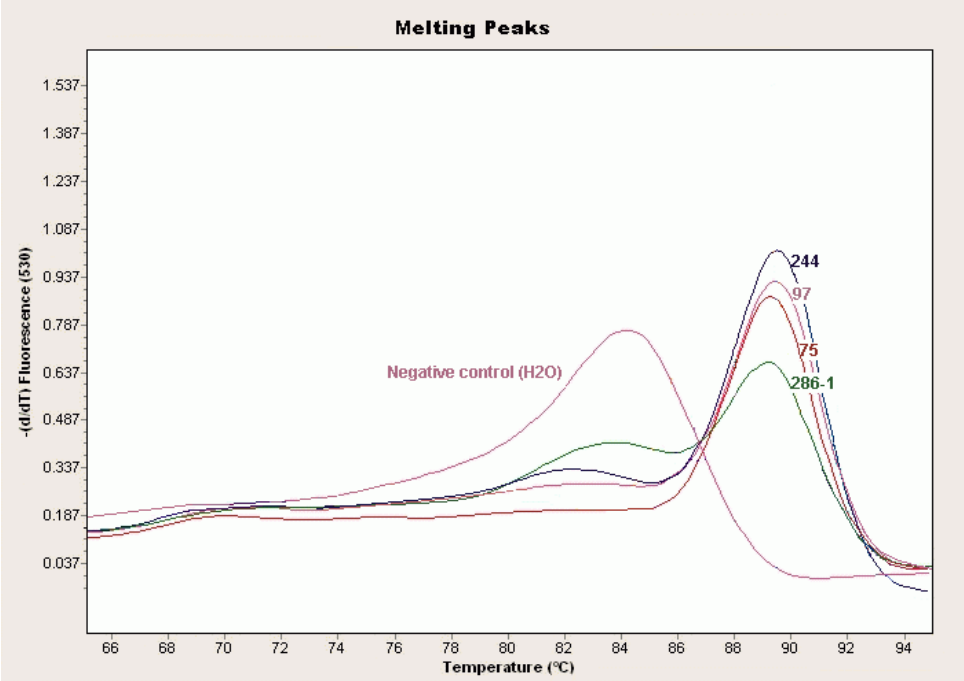

Figure 3.9: The melting curves of some of the positive samples (75, 97, 244 and 286–1) and the negative control, water.

The positive results were in accordance with the presence of a 274 bp band on agarose gel (Figure 3.10).

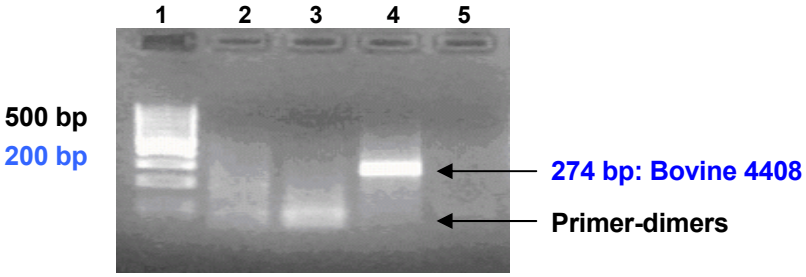

Figure 3.10: Agarose gel electrophoresis of the purified real-time PCR products. Lane 1 = DNA ladder. Lane 2 = sample 10-1. Lane 3 = sample 12-1. Lane 4 = Bovine 4408. Lane 5 = negative control (water). Bovine 4408 yielded a 274 bp band. The light bands visible at a size smaller than 100 bp are the primer-dimers that formed during the amplification process.

To evaluate the real-time PCR assay, 774 wildebeest samples (representing 304 animals and 51 foetuses) were tested. The samples consisted of EDTA blood (n=301), spleen (n=163) and

cornea (n=208) from black wildebeest culled or captured live. Foetal samples comprised of spleen (n=51) and cornea (n=51). Of the 774 samples tested, 106 samples (13.7%) tested positive for the presence of gammaherpesvirus DNA using the SYBR green real-time PCR assay (Table 3.1) (for complete results, see Appendix IV).

Table 3.1: Real-time PCR results of all the samples tested. Samples with a melting temperature within a 2°C range of either positive controls, Bovine 4408 and WB5, were noted as positive. Only positive results are listed in the table below (for complete results, see APPENDIX IV).

Lab ID "No"	Sample ID	Sex	Age	Samples			Foetal samples		
				EDTA blood	Spleen	Cornea	Spleen	Cornea	Sex
				No	No – 1	No – 2	No – 3	No – 4	
Tussen-die-Riviere TDR									
1	1	M	Adult	+					
4	4	F	Adult			+			
5	5	F	Adult			+			
8	8	M	Adult			+			
14	14	F	Sub-adult			+			
23	30	F	Adult				+		
27	34	F	Adult				+		
31	38	F	Adult				+		
34	41	F	Adult				+	+	
35	42	F	Adult					+	
38	45	F	Adult			+			
42	49	F	Adult					+	
43	50	F	Adult				+	+	
Schoondal TD									
55	112	M	Adult	+					
56	113	M	Adult	+					
Grootkoppe EE									
75	205	M	Juvenile	+					
86	216	M	Adult	+					
90	220	M	Juvenile	+					
97	227	M	Sub-adult	+					
Copperfield JF									
Maria Moroka MM									
142	408	F	Adult			+			
145	411	M	Adult			+			
147	413	M	Adult			+			
154	420	M	Adult			+			
164	430	M	Sub-adult			+			
167	433	F	Adult			+			
168	434	M	Adult			+			
169	435	M	Adult			+			
170	436	M	Adult			+			

Lab ID "No"	Sample ID	Sex	Age	Samples			Foetal samples		
				EDTA blood	Spleen	Cornea	Spleen	Cornea	Sex
				No	No – 1	No – 2	No – 3	No – 4	
173	439	F	Adult			+			
180	451	F	Adult				+	+	
182	453	F	Adult				+		
183	454	F	Adult					+	
184	455	F	Adult					+	
187	459	F	Juvenile		+	+			
188	460	F	Adult				+	+	
191	463	F	Adult					+	
192	464	F	Adult					+	
Gariep GP									
198	505	F	Adult				+		F
200	507	F	Adult				+		M
201	508	F	Adult				+		M
206	513	M	Adult				+		F
209	516	F	Sub-adult		+				
212	519	F	Adult			+	+		F
213	520	M	Adult		+	+			
215	522	F	Adult				+		F
217	524	F	Adult				+		F
219	526	F	Juvenile			+			
221	528	F	Adult				+		M
222	529	F	Adult				+	+	F
223	530	F	Adult				+		M
224	531	F	Adult				+		M
227	534	F	Adult			+	+		F
228	535	F	Adult			+	+		F
232	539	F	Adult		+				
233	540	M	Adult	+					
234	541	M	Juvenile			+			
235	542	F	Adult				+		M
236	543	F	Adult			+	+		F
237	544	F	Adult				+		F
238	545	F	Adult			+			
239	546	F	Adult				+		F
241	548	F	Adult				+		M
244	551	F	Adult	+			+		F
253	560	F	Adult				+		F
254	561	F	Adult				+		M
255	562	F	Adult				+		F
259	566	F	Adult				+		F
260	567	F	Adult				+		F
261	568	F	Adult				+		F
264	571	F	Adult				+		F
265	572	F	Adult				+		M
Sandveld									
268	602	M	Adult		+				
271	605	F	Adult		+				
272	606	F	Sub-adult		+				
273	607	F	Sub-adult		+				
274	608	F	Adult		+				

Lab ID "No"	Sample ID	Sex	Age	Samples			Foetal samples		
				EDTA	Spleen	Cornea	Spleen	Cornea	Sex
				No	No – 1	No – 2	No – 3	No – 4	
275	609	F	Adult		+		+	+	M
279	613	M	Adult		+				
282	616	M	Adult		+				
284	618	M	Adult			+			
286	620	M	Adult		+				
287	621	F	Adult				+	+	M
290	624	F	Adult				+		F
292	626	F	Adult		+				
297	631	F	Adult				+	+	M
298	632	F	Adult			+	+	+	M
300	634	F	Adult		+	+			

Foetal samples No samples taken Negative result

Note: Each animal is given a unique number for identification, i.e. the "No" (1 to 304). Each animal's spleen is therefore numbered according to this number as "No-1", the cornea as "No-2" and if a cow was pregnant, the foetal spleen as "No-3" and the foetal cornea as "No-4".

Of the 304 animals tested, only 48 animals (15.8%) yielded a positive test result from one or more of the samples collected from that animal (*i.e.* either EDTA blood, spleen and/or cornea) (Table 3.2, Figure 3.11).

Table 3.2: A summary of the number of black wildebeest samples collected and the number of real-time PCR positive results.

	Number of black wildebeest	EDTA blood samples	Spleen samples	Cornea samples	Total number of black wildebeest samples
Total number	304	301	163	208	672
Total positive	48	9	15	27	51
Percentage positive	15.8%	3.0%	9.2%	13.0%	7.6%

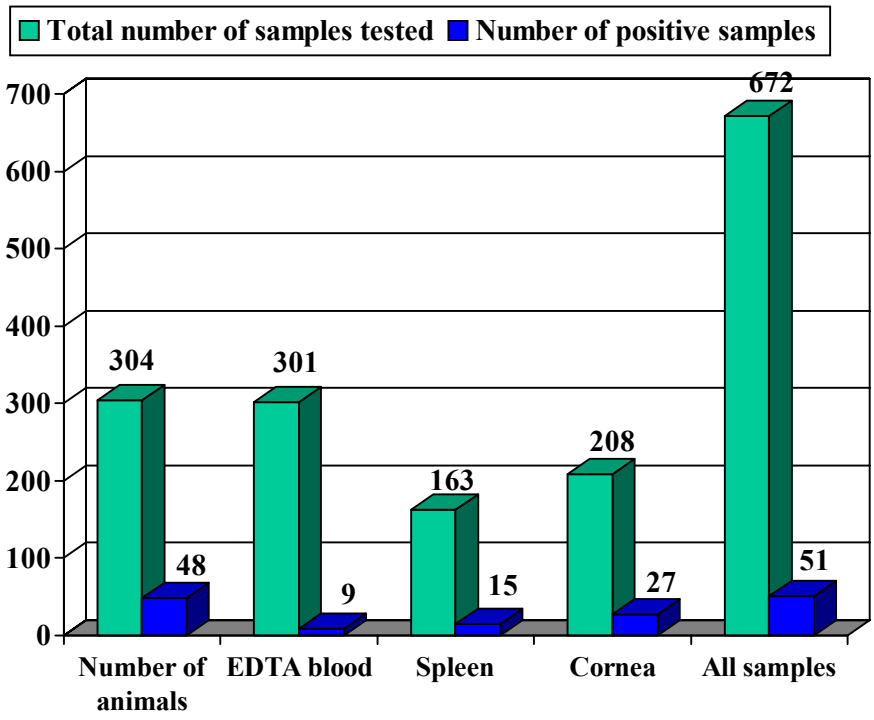

Figure 3.11: A summary of the total number of black wildebeest culled or captured and sampled, the total number of each type of sample collected and the number of positive samples.

Of the 51 foetuses, 46 foetuses (90.2%) yielded a positive result from either the spleen or cornea sample, or both (Table 3.3). Forty of the spleen samples (78.4%) tested positive, 15 corneas (29.4%) were positive and of these, nine were positive on both samples (Table 3.3, Figure 3.12). Five foetuses tested negative on both samples of which only one's dam yielded a weak positive result on her cornea sample.

Table 3.3: A summary of the number of black wildebeest foetal samples collected and the number of real-time PCR positive results.

	Number of foetuses	Foetal spleen samples	Foetal cornea samples	Total number of foetal samples
Total number	51	51	51	102
Total positive	46	40	15	55
Percentage positive	90.2%	78.4%	29.4%	53.9%

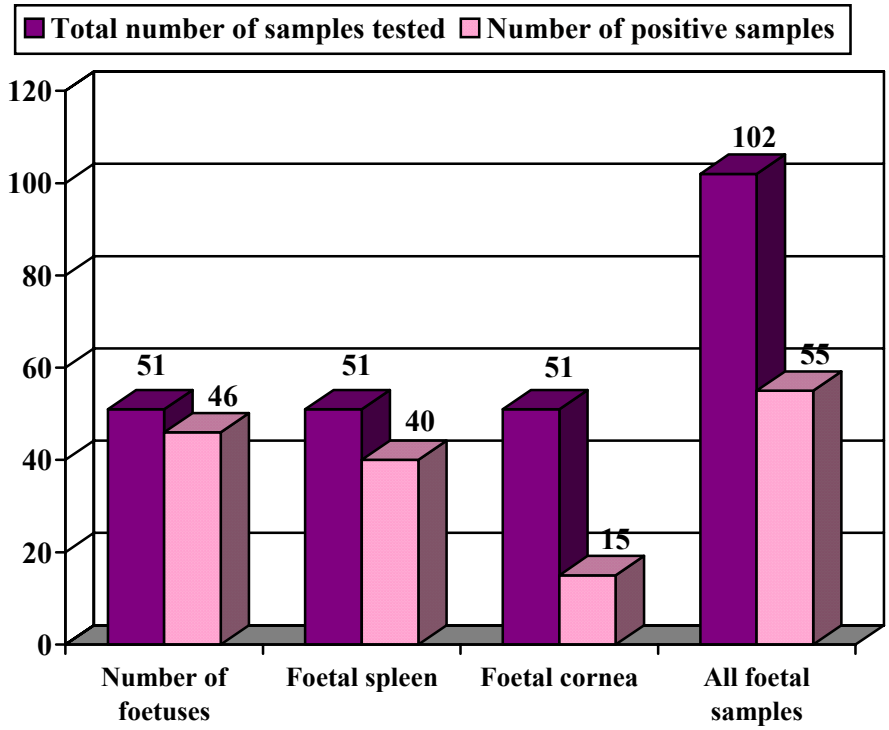

Figure 3.12: A summary of the total number of foetuses collected and sampled, the total number of each type of sample collected and the number of positive samples.

3.5 SEQUENCE ANALYSIS

The real-time PCR amplicons of controls Bovine 4408 and WB5 were sequenced to confirm the fragment amplified was the same as or similar to AlHV–1. The obtained sequences were edited to a 268 bp fragment and aligned with the published AlHV–1 genome sequence (Accession number: AF005370), as well as other published AlHV–1 published sequences (Figure 3.13). A phylogenetic tree was constructed using neighbor-joining in combination with the bootstrap method and is shown in Figure 3.14. A BLAST search revealed that the obtained WB5 sequence showed 93% homology to the published AlHV–1 genome. The sequence obtained for Bovine 4408 showed 98% homology to the published AlHV–1 genome.

The sequencing results showed a possible mixed infection, as the signal peaks in a few positions were similar in size (Figure 3.15). Depending on the percentage difference between these, it might suggest different strains or genotypes of the virus.

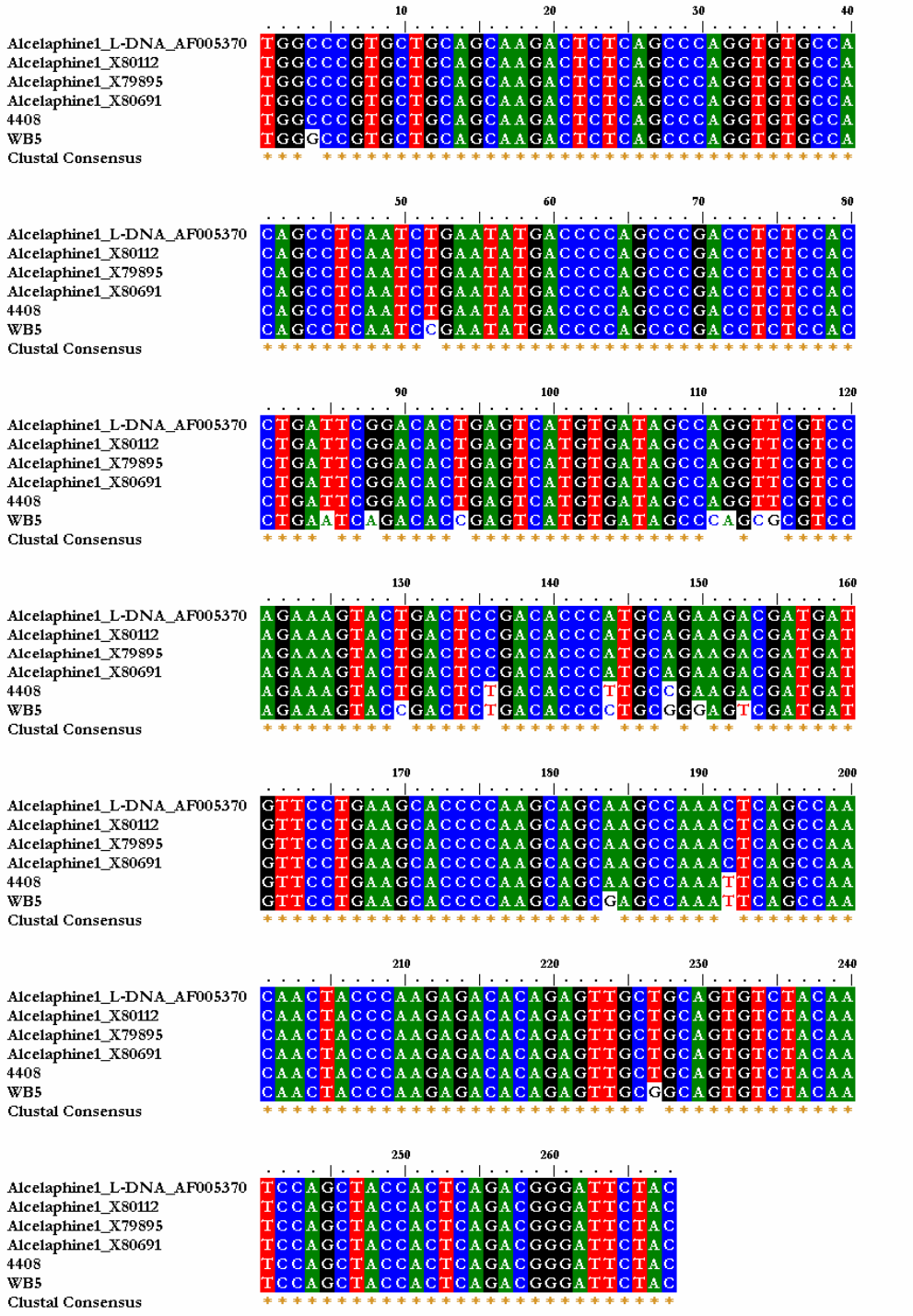

Figure 3.13: Alignment of the edited sequences of controls WB5 and Bovine 4408 with a section of the published AlHV–1 genome (AF005370) and other AlHV–1 published sequences.

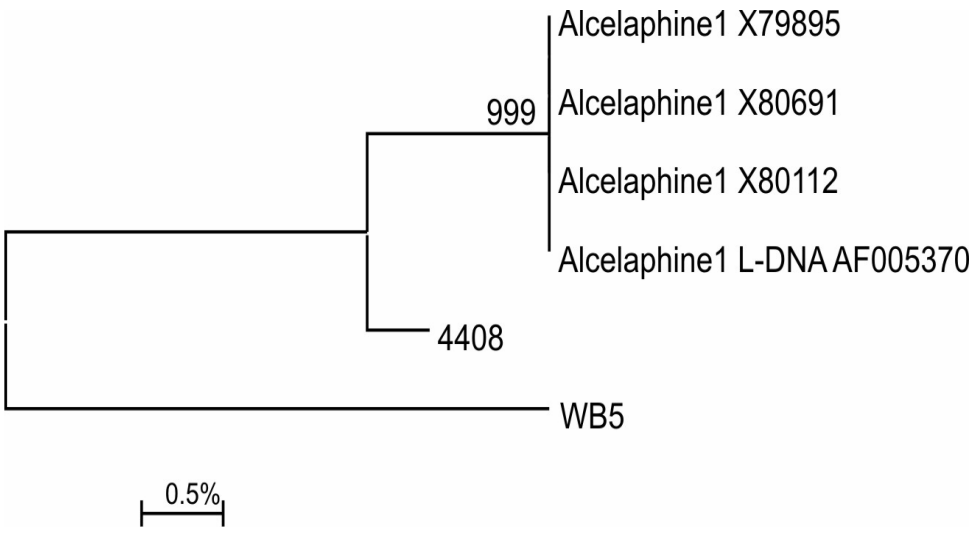

Figure 3.14: Neighbor-joining tree showing the phylogenetic relationship of WB5 and Bovine 4408 with AlHV–1, based on a 268 bp fragment of open reading frame 50. GenBank accession numbers are indicated on the tree. The scale bar represents the percentage nucleotide difference.

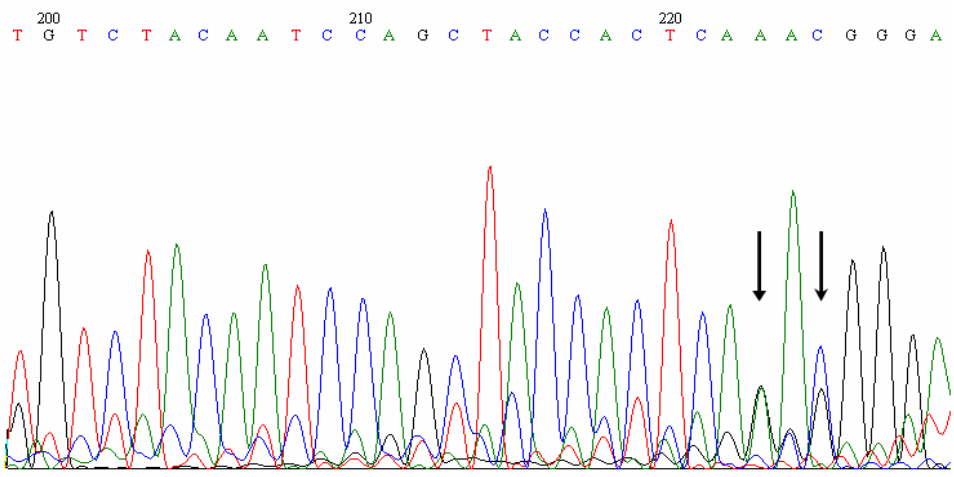

Figure 3.15: A section of the sequencing trace file as obtained from the virus isolated from WB5. Similar peaks at position 223 and 225, for example, could indicate a possible mixed infection.

Chapter 4

DISCUSSION

Alcelaphine herpesvirus type 1 has been studied widely in blue wildebeest but not in black wildebeest. As black wildebeest only occur naturally in the southern African region and very little is known about the virus carried by this species, a decision was made to study the gammaherpesvirus carrier status in this species.

The black wildebeest samples originated from a wide geographical area in South Africa. The different sampling areas stretch from 26°S to 31°S and 24°E to 30°E. Some of these populations may originally have been from the same areas but have been separated for several generations.

4.1 SEROLOGY

The 269 black wildebeest serum samples that were tested for MCF antibodies using the CI-ELISA kit, all tested positive. This indicates that the virus is maintained in the populations and spreads to newborn calves either before or soon after birth. Congenital transmission seems to be the most likely route of infection, judging by the fact that 46 of 51 foetuses (90.2%) yielded positive real-time PCR results. It is known that herpesviruses cause persistent infections and even though clinical disease or shedding of virus may be absent, animals remain carriers for life. A positive serological result indicates a persistent infection even though the viral load might be so low that it is not detectable by PCR assay. Although this CI-ELISA is said to be less sensitive than histopathology and PCR to diagnose MCF in clinically affected animals[17], this may only be a disadvantage when the aim is to test acutely ill animals such as

cattle, deer or bison. In acutely ill animals, the antibody response may not have reached detectable levels, compared to the natural host in which it is a lifelong infection without development of clinical signs. The CI-ELISA is, however, very specific with a specificity that ranges between 91 and 100%[17].

The buffalo bull (BB) that spent six months in the holding pens with a group of black wildebeest before they were relocated, did not seroconvert even though buffaloes are known to be susceptible to MCF, especially when in captivity and under stressful conditions. The black wildebeest calves in the holding pens were, however, all weaned and probably not excreting cell-free infective virus during that period.

The serological results represent the first documented confirmation of black wildebeest as carriers of a gammaherpesvirus antigenically similar to or closely related to AlHV–1. The black wildebeest sampled represented an extensive geographical range and 100% of the tested population were positive.

4.2 VIRAL ISOLATION AND IDENTIFICATION

Virus is excreted by the natural host, the wildebeest, in a cell-free form for a brief period following infection and can be isolated from nasal swabs and blood leukocytes during that period[33]. Viral isolation from clinically affected animals can be achieved from cell suspensions of either peripheral blood leukocytes, lymph nodes or other affected tissues[33].

The virus isolated from the black wildebeest calf WB5 was confirmed by electron microscopy to be a herpesvirus. The size of approximately 100 nm, the icosahedral capsid and tegument were compatible with typical herpesvirus morphology. Based on the

morphological, phenotypic, antigenic and genetic characteristics, it was found to be a gammaherpesvirus closely related to AlHV–1.

4.3 CONVENTIONAL PCR

Four different conventional PCR assays previously described were evaluated. The aim was to find an assay suitably sensitive and specific enough to test the black wildebeest samples for the presence of a gammaherpesvirus similar or related to AlHV–1. This was important as it was shown by the CI-ELISA that all the animals tested were carriers. All four PCR assays gave poor and inconsistent results. In almost all the tests done no PCR products could be visualised on agarose gel, except for primer-dimers that had formed.

The control Bovine 4408 was tested several times by using the assay described by Murphy *et al*[42]. It yielded only one positive result in all of the assays done. The primers described by Murphy *et al*[42] were aligned with the published AlHV–1 genome sequence (Accession number AF005370[14] – data not shown). It was found that the primers amplified an area spanning genes 22 and 23. This is probably not an ideal set of primers as the area between two genes is very often highly variable. The assay was developed by the authors to test clinically sick animals for AlHV–1 and was evaluated on an Indian gaur that became ill in a zoo[42]. In clinically affected animals, circulating viral levels are high and more easily detectable by PCR assay than in carrier animals. Murphy *et al*[42] also used Southern blot hybridisation to confirm the identity of the DNA bands after PCR amplification, which was not done in this study.

VanDevanter *et al*[69] described a nested format in which primary and secondary PCRs are performed with degenerate primers, targeting highly conserved amino acid motifs within an approximately 800 bp

region of the herpesviral-directed DNA polymerase gene. This PCR was developed to detect and identify known herpesviruses in clinical samples, as well as to characterise new herpesviral genomes. According to the authors, this assay successfully amplifies a 215 to 315 bp region of 21 out of 22 different herpesviral species tested. The virus that did not amplify in their study lacked the amino acid motifs used to design the primers. The study did not include AlHV–1 and therefore the virus may also lack the specific amino acid motifs. This could explain why the control, Bovine 4408, did not yield any PCR amplicons that could be visualised on agarose gel. In a study by Li et al[25] to identify the virus causing MCF in white-tailed deer, he also had difficulty amplifying the viral sequences from spleen samples by using the abovementioned degenerate primers. The author developed more specific degenerate primers from the known polymerase gene sequences obtained from AlHV–1 and OvHV–2 that they successfully used to amplify viral sequences[25].

The assay described by Flach *et al*[16] was developed to test different species of the order *Artiodactyla* for evidence of infection with a gammaherpesvirus to distinguish between species susceptible to MCF caused by AlHV–1 or OvHV–2 and species that are carriers of a related virus subclinically. This study yielded many inconsistent results with the AlHV–Pol and OvHV–Pol PCRs. This would suggest that the black wildebeest virus is related to but distinct from AlHV–1 and OvHV–2. The other possible reason and likely applicable to all the different primer sets, is the fact that the viral load is just too low to be detected by conventional PCR.

The same applies to the primers described by Li *et al*[25]. The assay was developed to detect the MCF-causing virus in white-tailed deer. The primers were however derived from the sequence of AlHV–1

open reading frame 50, a region associated with virulence in rabbits and did not amplify any viral sequences from the white-tailed deer. In the present study, none of the samples, including control Bovine 4408, tested positive with this conventional PCR.

Based on the above, several reasons can be listed to justify why the conventional PCR assays used gave poor and inconsistent results with the black wildebeest samples: (i) Primers may not be specific enough for the relevant virus that is tested for, especially if the virus is fairly unknown. An assumption can be made that the virus carried by black wildebeest is similar to the virus carried by blue wildebeest because these two species are closely related and are both associated with the spread of malignant catarrhal fever virus to cattle. The published AlHV–1 genome was, however, derived from sequencing a virus isolated from blue wildebeest. It is known that OvHV–2 and AlHV–1 differ genetically but both cause MCF in cattle, deer, bison and other antelope species. Preliminary sequencing results point to the fact that the viruses from black and blue wildebeest may have sequence differences. (ii) The viral load in the blood or sampled organs could be very low as is often the case in carrier animals. Low DNA input concentration may lead to insufficient amplification by conventional PCR. The concentration of the amplified product may consequently be too low to be visualised under UV-light on agarose gel. (iii) Handling and storage of samples also play an important role. If samples are incorrectly handled, exposed to high temperatures and stored at incorrect temperatures, DNA undergo degradation with no or poor amplification. (iv) The assay itself may not be sensitive or specific enough, or optimised properly. Several factors can be responsible, such as incorrect temperature settings, primers that are not specific enough; or the

concentrations of the primers, input DNA or magnesium chloride might be insufficient.

The four conventional PCR assays investigated in this study were unable to amplify viral DNA from black wildebeest samples and gave inconsistent results. The question would be whether the assays tested were optimised to amplify the specific viral DNA in black wildebeest. In the respective publications describing the different PCR assays, it performed well in other species but was not adequate for the black wildebeest samples tested in this project. It can be concluded that conventional PCR was not sensitive enough for the black wildebeest samples tested in this project, as it likely had very low viral numbers.

4.4 REAL–TIME PCR

A SYBR Green real-time PCR assay was developed and evaluated using the nested primers as described by Li *et al*[25]. Real-time PCR is more sensitive than conventional PCR and any products formed are easily visualised using the LightCycler software. Low input DNA concentrations can be adequately amplified by real-time PCR to be analysed and visualised by computer software. Melting curve analysis was used to increase the specificity of the real-time PCR assay by confirming that a positive fluorescence signal at 530 nm is associated with an amplified product with a characteristic T_m. Melting curves can also be generated at very low concentrations of amplified product, increasing sensitivity.

In this study the T_m of the positive controls, Bovine 4408 and WB5, differed by 0.71°C on average throughout all the assays with the smallest difference being 0.55°C and the biggest difference 1.57°C (Appendix III). This most likely reflects the genetic variation in this

region of ORF 50 between virus types present. Bovine 4408 died of MCF related to blue wildebeest, whereas WB5 is virus isolated from a black wildebeest calf. Genetic variation as reason for the difference in the melting temperatures (T_m) was supported by the sequence differences found between WB5 and Bovine 4408. The theoretical T_m of the fragments amplified of the control fragments amplified can be determined by determining the percentage GC content. WB5 has a 56.7% GC content and a theoretical T_m of 86°C. The other control, Bovine 4408, has a GC content of 53.7% and this gives a theoretical T_m of 84°C. This difference is displayed in the real-time PCR assays with the melting temperature of Bovine 4408 always lower than WB5. The higher the GC content, the higher the T_m as a result of stronger hydrogen bonds between G and C nucleotides.

It is, however, possible that run-to-run differences and in-run differences can result in up to a 2°C variation between samples and without further sequence analysis, this cannot completely be excluded as a reason for the variation. The percentage differences in base pairs will be investigated by further sequencing of the virus isolated from the black wildebeest calf WB5.

The melting temperatures of the black wildebeest samples that tested positive were almost all between 0.1°C and 1.3°C higher than the melting temperature of WB5 in each run. Only four samples had a melting temperature between 0.1°C and 1.1°C lower than WB5 but still higher than Bovine 4408.

Only 106 of 774 (13.7%) black wildebeest samples (blood in EDTA, spleen, cornea and foetal samples) tested positive using real-time PCR. This may be due to several reasons. The two controls, the

virus isolated from black wildebeest calf WB5 and the bovine that died of MCF, Bovine 4408, tested positive with every LightCycler run. The viral input DNA in both these would be higher than field samples from carrier animals. DNA input can therefore be increased as one possible way of increasing sensitivity. The choice of samples may need to be re-evaluated as the virus may be more concentrated in other organs, for example in lymph nodes and the central nervous system[33]. Working with only EDTA blood, spleen and cornea, only 15.8% (48 of 304) of the animals tested positive on one or more samples. Three percent (9 of 301) of the blood samples tested positive; 9.3% (15 of 163) of the spleen samples and 13% (27 of 208) of the cornea samples tested positive (Table 3.2).

The majority of animals that tested positive were culled during the last part of the winter and early spring before the first rains for the season. Nutritional stress may be the reason for the higher numbers of circulating virus at this time of year. Of the 48 positive animals, 13 were from Sandveld Nature Reserve and were culled during September 2005. In total, 38 animals were culled there which indicates that 34.2% of animals culled on this reserve had circulating virus detectable with real-time PCR. Most of the positive results were from spleen samples.

The Gariep Nature Reserve had 12 positive animals that were culled during August 2005. Twelve of 73 animals tested positive indicating that 16.4% had high enough circulating virus, although in this case the positive results were equally distributed between the spleen and cornea. Black wildebeest were culled in June 2005 on Maria Moroka Reserve and 11 of 59 animals culled tested positive on real-time PCR, yielding 18.6% positive animals, diagnosed mainly on the cornea samples. The other reserves had between 0 and 14%

positive animals with the real-time PCR but on three of these reserves (Schoondal – 7.4%, Grootkoppe – 12.5%, and Copperfield – 0%), only live animals were caught and therefore only blood samples collected. Tussen-die-Riviere Nature Reserve had 6 of 43 (14.0%) animals testing positive with real-time PCR and samples were taken during April, May and June 2005. Most of the animals that tested positive were adult animals but this portion of the population was over represented as culling operations focused on the adult animals for meat harvesting purposes. Foetuses testing positive on real-time PCR were evenly distributed between the reserves where animals were culled.

The real-time PCR results confirmed that foetuses already become infected *in utero*. Ninety percent (90%) of the foetuses tested positive on one or both of the cornea and spleen samples. Only five of the 51 foetuses (9.8%) tested negative on both samples with real-time PCR. The results showed that unlike sheep lambs, most of the black wildebeest calves are infected *in utero*. Of the 51 foetuses, 78.4% (40 of 51) had positive spleen samples, 29.4% (15 of 51) had positive cornea samples and of these, 19.6% (9 of 46 positive foetuses) or 17.7% (9 of 51 foetuses) were positive with both tissue types.

Keeping in mind that all of the serum samples tested positive for antibodies, there can be many reasons for the low number of positive real-time PCR results. Extremely low viral numbers in the samples is probably the main reason. If a virus is in a latent form and only starts replicating under stressful and immune-compromising situations, the animal's samples might be taken at the time when the viral load is at its lowest point. In addition, the type of

sample may play a role and other tissue samples may give better results. This needs to be investigated in future studies.

4.5 SEQUENCE ANALYSIS

After electron microscopy confirmed the isolated virus to be a herpesvirus, further confirmation was necessary that the virus under consideration was indeed a gammaherpesvirus. The DNA fragments from the gene ORF 50 of the two control viruses, WB5 and Bovine 4408, were sequenced, edited and aligned to the published AlHV–1 genome (Accession number AF005370[14]). A BLAST search revealed that the sequence of WB5 was 93.3% similar to the published AlHV–1 genome. The sequence of Bovine 4408 revealed a 98.5% similarity to the published AlHV–1 genome. There is a 6% nucleotide difference between WB5 and Bovine 4408. Taking into consideration possible errors that can occur with sequencing, it was confirmed that WB5 was closely related to AlHV–1. It was compared to other published sequences as well and no other mammalian virus was found to be similar.

The sequencing results, however, showed a possible mixed infection. The signals were mixed but clear and similar enough to rule out contamination of the sample. The peaks were exactly over one another, implying similar and very closely related sequences. Searching GenBank for similar sequences that could have caused contamination did not produce any results. The possibility exists that the virus in black and blue wildebeest may differ slightly as these two species are historically from different regions. The published genome was sequenced from viral isolates of blue wildebeest. In recent years, the two species have been kept on the same properties and even though this practice has been stopped, mixed infections might occur through contact between these animals. The

possibility therefore exists that the black and blue wildebeest may either carry their own strains or genotypes of the virus or that both species carry several different strains or genotypes of the virus. This will be investigated in a further study by cloning the PCR products in vectors and sequencing individual PCR amplicons.

Chapter 5

CONCLUSIONS

Black wildebeest are all latent carriers of a gammaherpesvirus similar or related to AlHV–1 present in blue wildebeest. Positive serological test results of all tested serum samples confirm the theory that all black wildebeest are carriers of a malignant catarrhal fever-causing virus. Conventional PCR does not seem to be sensitive enough for the low viral numbers in the blood, spleen or cornea. The increased sensitivity of real-time PCR and analysis of very low concentrations of amplified product makes it a better choice of assay than conventional PCR. The assay can further be improved by possibly using sequence specific probes. It is, however, necessary to further investigate the sequence of the virus isolated from the black wildebeest calf WB5, as more sequence data is necessary to develop better assays. This information is also important to determine whether the black and blue wildebeest carry their own strains or genotypes or have mixed infections. In future, investigation of a wider variety of samples will be needed, as the virus may be more concentrated in other organs.

A very important fact that emerged is that 90% of the foetuses are infected *in utero*. This would suggest that unlike sheep, black wildebeest calves shed most virus soon after birth when titres are still high. A follow-up study that will investigate the viral shedding patterns from newborn black wildebeest calves can be achieved using quantitative real-time PCR technology. Further sequencing and genetic analysis of the gammaherpesvirus carried by black wildebeest is also important for diagnostic and research purposes.

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APPENDICES

APPENDIX I: SAMPLE LIST

2005	Lab ID	Sample ID	Sex	Age	Foetus	Size	Sex
Tussen-die-Riviere	**WD**	**TDR**					
19-Apr	1	1	M	Adult			
	2	2	F	Sub-adult			
	3	3	M	Adult			
	4	4	F	Adult			
	5	5	F	Adult			
	6	6	F	Sub-adult			
	7	7	F	Adult			
	8	8	M	Adult			
	9	9	F	Adult			
	10	10	F	Adult			
	11	11	F	Adult			
	12	12	F	Sub-adult			
	13	13	F	Sub-adult			
	14	14	F	Sub-adult			
10-May	15	20	M	Adult			
	16	21	F	Adult			
	17	22	F	Adult			
	18	23	F	Adult			
	19	24	M	Adult			
	20	25	F	Adult			
13-Jun	21	28	F	Adult			
	22	29	F	Adult			
	23	30	F	Adult	Y	14-15cm	
	24	31	F	Juvenile			
	25	32	F	Adult			
	26	33	F	Adult	Y	14-15cm	
	27	34	F	Adult	Y	14-15cm	
	28	35	F	Adult			
	29	36	F	Adult			
	30	37	F	Adult			
	31	38	F	Adult	Y	14-15cm	
	32	39	F	Adult			
	33	40	F	Juvenile			
	34	41	F	Adult	Y	14-15cm	
	35	42	F	Adult	Y	14-15cm	
	36	43	F	Sub-adult			
	37	44	F	Juvenile			
	38	45	F	Adult	Y	14-15cm	
	39	46	F	Sub-adult			
	40	47	M	Adult			
	41	48	M	Adult			
	42	49	F	Adult	Y	14-15cm	
	43	50	F	Adult	Y	14-15cm	
Schoondal	**WD**	**TD**					
29-Apr	44	101	M	Adult			
	45	102	M	Adult			
	46	103	M	Adult			
	47	104	M	Adult			

2005	Lab ID	Sample ID	Sex	Age	Foetus	Size	Sex
	48	105	M	Adult			
	49	106	M	Adult			
	50	107	M	Adult			
	51	108	M	Adult			
	52	109	M	Adult			
	53	110	M	Adult			
	54	111	M	Adult			
	55	112	M	Adult			
	56	113	M	Adult			
	57	114	M	Adult			
	58	115	M	Adult			
	59	116	M	Adult			
	60	117	M	Adult			
	61	118	M	Adult			
	62	119	F	Sub-adult			
	63	120	M	Sub-adult			
	64	121	M	Sub-adult			
	65	122	F	Sub-adult			
	66	123	M	Adult			
	67	124	F	Adult			
	68	125	F	Adult			
	69	126	F	Adult			
	70	127	M	Juvenile			
Grootkoppe	**WD**	**EE**					
06-May-05	71	201	M	Juvenile			
	72	202	F	Adult			
	73	203	F	Adult			
	74	204	M	Adult			
	75	205	M	Juvenile			
	76	206	F	Adult			
	77	207	F	Adult			
	78	208	F	Adult			
	79	209	F	Adult			
	80	210	M	Juvenile			
	81	211	F	Juvenile			
	82	212	F	Juvenile			
	83	213	F	Adult			
	84	214	F	Juvenile			
	85	215	F	Adult			
	86	216	M	Adult			
	87	217	F	Adult			
	88	218	M	Juvenile			
	89	219	F	Adult			
	90	220	M	Juvenile			
	91	221	F	Adult			
	92	222	F	Adult			
	93	223	F	Adult			
	94	224	F	Adult			
	95	225	F	Juvenile			
08-May-05	96	226	M	Adult			
	97	227	M	Sub-adult			
	98	228	M	Adult			
	99	229	M	Adult			
	100	230	M	Adult			
	101	231	M	Adult			
	102	232	M	Adult			

2005	Lab ID	Sample ID	Sex	Age	Foetus	Size	Sex
Copperfield	**WD**	**JF**					
16-May-05	103	301	F	Sub-adult			
	104	302	F	Juvenile			
	105	303	M	Adult			
	106	304	M	Adult			
	107	305	M	Adult			
	108	306	M	Adult			
	109	307	M	Adult			
	110	308	M	Adult			
	111	309	M	Adult			
	112	310	M	Adult			
	113	311	M	Adult			
	114	312	M	Adult			
	115	313	M	Adult			
	116	314	M	Adult			
	117	315	F	Sub-adult			
	118	316	F	Adult			
	119	317	F	Juvenile			
	120	318	F	Adult			
	121	319	F	Adult			
	122	320	F	Adult			
	123	321	F	Adult			
	124	322	F	Adult			
	125	323	F	Adult			
	126	324	F	Adult			
	127	325	F	Adult			
	128	326	F	Juvenile			
	129	327	F	Adult			
	130	328	F	Juvenile			
	131	329	F	Adult			
	132	330	M	Juvenile			
	133	331	F	Juvenile			
	134	332	M	Juvenile			
Maria Moroka	**WD**	**MM**					
06-Jun	135	401	M	Adult			
	136	402	M	Adult			
	137	403	M	Sub-adult			
	138	404	M	Adult			
	139	405	M	Sub-adult			
	140	406	M	Adult			
	141	407	M	Adult			
	142	408	F	Adult			
	143	409	M	Sub-adult			
	144	410	M	Adult			
	145	411	M	Adult			
	146	412	F	Adult			
	147	413	M	Adult			
	148	414	M	Adult			
	149	415	M	Adult			
	150	416	F	Adult			
	151	417	M	Adult			
	152	418	F	Adult			
	153	419	M	Adult			
	154	420	M	Adult			
	155	421	F	Adult			
	156	422	M	Adult			
	157	423	F	Adult			

2005	Lab ID	Sample ID	Sex	Age	Foetus	Size	Sex
	158	424	F	Adult			
	159	425	M	Adult			
	160	426	F	Adult			
	161	427	F	Adult			
	162	428	M	Adult			
	163	429	F	Adult			
	164	430	M	Sub-adult			
	165	431	F	Adult			
	166	432	F	Adult			
	167	433	F	Adult			
	168	434	M	Adult			
	169	435	M	Adult			
	170	436	M	Adult			
	171	437	F	Adult			
	172	438	F	Sub-adult			
	173	439	F	Adult			
	174	440	F	Juvenile			
	175	441	F	Adult			
	176	442	F	Adult			
	177	443	F	Adult			
	178	444	M	Adult			
	179	445	F	Juvenile			
	180	451	F	Adult	Y	20cm	
	181	452	M	Juvenile			
	182	453	F	Adult	Y	18cm	
	183	454	F	Adult	Y	21cm	
	184	455	F	Adult	Y	20cm	
	185	457	M	Adult			
	186	458	M	Juvenile			
	187	459	F	Juvenile			
	188	460	F	Adult	Y	15cm	
	189	461	M	Adult			
	190	462	M	Juvenile			
	191	463	F	Adult	Y	20cm	
	192	464	F	Adult	Y	15cm	
	193	465	F	Juvenile			
Gariep	**WD**	**GP**					
03-Aug	194	501	M	Adult			
	195	502	F	Sub-adult			
	196	503	M	Adult			
	197	504	F	Sub-adult			
	198	505	F	Adult	Y	30cm	F
	199	506	M	Adult			
	200	507	F	Adult	Y	32cm	M
	201	508	F	Adult	Y	32cm	M
	202	509	M	Adult			
	203	510	M	Adult			
	204	511	M	Adult			
	205	512	M	Adult			
	206	513	M	Adult	Y	31cm	F
	207	514	M	Juvenile			
	208	515	F	Adult			
	209	516	F	Sub-adult			
	210	517	F	Sub-adult			
	211	518	F	Adult			
	212	519	F	Adult	Y	20cm	F
	213	520	M	Adult			

2005	Lab ID	Sample ID	Sex	Age	Foetus	Size	Sex
	214	521	M	Adult			
	215	522	F	Adult	Y	31cm	F
	216	523	M	Adult			
	217	524	F	Adult	Y	34cm	F
	218	525	F	Sub-adult			
	219	526	F	Juvenile			
	220	527	M	Adult			
	221	528	F	Adult	Y	35cm	M
	222	529	F	Adult	Y	24cm	F
	223	530	F	Adult	Y	29cm	M
	224	531	F	Adult	Y	34cm	M
	225	532	F	Juvenile			
	226	533	M	Adult			
	227	534	F	Adult	Y	30cm	F
	228	535	F	Adult	Y	30cm	F
	229	536	F	Juvenile			
	230	537	F	Adult			
	231	538	M	Adult			
	232	539	F	Adult			
	233	540	M	Adult			
	234	541	M	Juvenile			
	235	542	F	Adult	Y	30cm	M
	236	543	F	Adult	Y	32cm	F
	237	544	F	Adult	Y	26cm	F
	238	545	F	Adult			
	239	546	F	Adult	Y	34cm	F
	240	547	F	Sub-adult			
	241	548	F	Adult	Y	16cm	M
	242	549	M	Adult			
	243	550	F	Adult	Y	40cm	F
	244	551	F	Adult	Y	40cm	F
	245	552	F	Sub-adult			
	246	553	M	Sub-adult			
	247	554	M	Adult			
	248	555	F	Adult			
	249	556	M	Adult			
	250	557	M	Adult			
	251	558	F	Sub-adult			
	252	559	M	Sub-adult			
	253	560	F	Adult	Y	39cm	F
	254	561	F	Adult	Y	28cm	M
	255	562	F	Adult	Y	34cm	F
	256	563	M	Juvenile			
	257	564	F	Juvenile			
	258	565	F	Adult			
	259	566	F	Adult	Y	32cm	F
	260	567	F	Adult	Y	31cm	F
	261	568	F	Adult	Y	30cm	F
	262	569	F	Adult			
	263	570	M	Adult			
	264	571	F	Adult	Y	30cm	F
	265	572	F	Adult	Y	33cm	M
Thaba Manzi	266	Thaba Manzi	F	Juvenile			

2005	Lab ID	Sample ID	Sex	Age	Foetus	Size	Sex
Sandveld	WD	SAV					
19-Sep	267	601	M	Adult			
	268	602	M	Adult			
	269	603	M	Adult			
	270	604	M	Adult			
	271	605	F	Adult			
	272	606	F	Sub-adult			
	273	607	F	Sub-adult			
	274	608	F	Adult			
	275	609	F	Adult	Y	56cm	M
	276	610	M	Adult			
	277	611	M	Adult			
	278	612	M	Adult			
	279	613	M	Adult			
	280	614	M	Adult			
	281	615	M	Adult			
	282	616	M	Adult			
20-Sep	283	617	F	Adult			
	284	618	M	Adult			
	285	619	F	Adult			
	286	620	M	Adult			
	287	621	F	Adult	Y	43cm	M
	288	622	M	Sub-adult			
	289	623	F	Sub-adult			
	290	624	F	Adult	Y	49cm	F
	291	625	F	Adult			
	292	626	F	Adult			
	293	627	M	Sub-adult			
	294	628	F	Adult	Y	41cm	F
	295	629	F	Adult			
	296	630	F	Sub-adult			
	297	631	F	Adult	Y	59cm	M
	298	632	F	Adult	Y	49cm	M
	299	633	F	Adult			
	300	634	F	Adult			
	301	635	F	Adult			
	302	636	M	Adult			
	303	637	M	Adult			
	304	638	F	Adult	Y	56cm	M

APPENDIX II: SEROLOGICAL RESULTS

Plate 1	Well number	Lab ID	Sex	Age	Percentage inhibition	Result
Tussen-die-Riviere TDR	C1	2	F	Adult	84.644	Positive
	D1	3	M	Adult	58.413	Positive
	E1	4	F	Adult	90.007	Positive
	F1	5	F	Adult	91.697	Positive
	H1	6	F	Sub-adult	87.583	Positive
	A2	8	M	Adult	91.256	Positive
	B2	9	F	Adult	92.285	Positive
	C2	10	F	Adult	87.877	Positive
	D2	12	F	Sub-adult	85.893	Positive
	E2	13	F	Adult	91.624	Positive
	F2	14	F	Adult	92.579	Positive
	G2	23	F	Adult	88.611	Positive
	H2	24	F	Juvenile	91.991	Positive
	A3	26	F	Adult	91.109	Positive
	B3	27	F	Adult	89.860	Positive
	C3	28	F	Adult	92.506	Positive
	D3	29	F	Adult	87.950	Positive
	E3	30	F	Adult	88.758	Positive
	F3	31	F	Adult	91.330	Positive
	G3	32	F	Adult	87.289	Positive
	H3	34	F	Adult	87.803	Positive
	A4	35	F	Adult	90.081	Positive
	B4	36	F	Sub-adult	84.129	Positive
	C4	37	F	Juvenile	92.873	Positive
	D4	38	F	Adult	89.420	Positive
	E4	39	F	Sub-adult	92.799	Positive
	F4	40	M	Adult	87.730	Positive
	G4	41	M	Adult	79.868	Positive
	H4	42	F	Adult	90.669	Positive
	A5	43	F	Adult	90.963	Positive
Schoondal TD	B5	44	M	Adult	84.864	Positive
	C5	45	M	Adult	84.938	Positive
	D5	46	M	Adult	50.184	Positive
	E5	47	M	Adult	86.040	Positive
	F5	48	M	Adult	70.977	Positive
	G5	49	M	Adult	84.203	Positive
	H5	50	M	Adult	84.276	Positive
	A6	51	M	Adult	92.726	Positive
	B6	52	M	Adult	91.403	Positive
	C6	53	M	Adult	77.223	Positive
	E6	54	M	Adult	91.624	Positive
	F6	55	M	Adult	92.799	Positive
	G6	56	M	Adult	85.305	Positive
	H6	57	M	Adult	90.228	Positive
	A7	58	M	Adult	78.545	Positive

	B7	59	M	Adult	92.726	Positive
	C7	61	M	Adult	88.979	Positive
	D7	63	M	Sub-adult	81.264	Positive
	F7	64	M	Sub-adult	90.448	Positive
	G7	70	M	Juvenile	91.036	Positive
Grootkoppe EE	H7	71	M	Juvenile	86.334	Positive
	A8	78	F	Adult	90.301	Positive
	B8	79	F	Adult	91.477	Positive
	C8	80	M	Juvenile	92.726	Positive
	D8	81	F	Juvenile	92.432	Positive
	E8	82	F	Juvenile	92.873	Positive
	F8	83	F	Adult	90.742	Positive
	G8	84	F	Juvenile	92.579	Positive
	H8	88	M	Juvenile	90.007	Positive
	A9	89	F	Adult	82.145	Positive
	B9	90	M	Juvenile	93.387	Positive
	C9	93	F	Adult	91.697	Positive
	D9	94	F	Adult	88.758	Positive
	E9	95	F	Juvenile	92.652	Positive
	F9	96	M	Adult	87.730	Positive
	G9	98	M	Adult	92.506	Positive
	H9	99	M	Adult	88.832	Positive
	A10	101	M	Adult	88.464	Positive
	B10	102	M	Adult	91.183	Positive
Thaba Manzi TM	C10	Buffalo	M	Sub-adult	1.470	Negative
Copperfield JF	D10	103	F	Sub-adult	85.452	Positive
	E10	104	F	Juvenile	90.889	Positive
	F10	105	M	Adult	92.726	Positive
	G10	106	M	Adult	92.579	Positive
	H10	107	M	Adult	87.803	Positive
	A11	108	M	Adult	88.538	Positive
	B11	109	M	Adult	91.403	Positive
	C11	110	M	Adult	92.726	Positive
	D11	112	M	Adult	87.656	Positive
	E11	113	M	Adult	90.669	Positive
	F11	116	M	Adult	82.513	Positive
	G11	118	F	Adult	64.585	Positive
	H11	119	F	Juvenile	87.289	Positive
	A12	120	F	Adult	89.346	Positive
	C12	122	F	Adult	82.292	Positive
	D12	126	F	Adult	88.097	Positive
	E12	131	F	Adult	90.301	Positive
	F12	132	M	Juvenile	90.228	Positive
	G12	133	F	Juvenile	88.464	Positive
	H12	134	M	Juvenile	83.909	Positive

Plate 2	Well number	Lab ID	Sex	Age	Percentage inhibition	Result
Maria Moroka MM	C1	135	M	Adult	86.511	Positive
	D1	136	M	Adult	81.777	Positive
	E1	137	M	Sub-adult	84.176	Positive
	F1	138	M	Adult	86.511	Positive
	H1	140	M	Adult	82.685	Positive
	A2	141	M	Adult	86.965	Positive
	B2	142	F	Adult	86.965	Positive
	C2	148	M	Adult	86.511	Positive
	D2	152	F	Adult	87.938	Positive
	E2	153	M	Adult	84.112	Positive
	F2	154	M	Adult	89.429	Positive
	G2	155	F	Adult	89.300	Positive
	H2	159	M	Adult	86.835	Positive
	A3	163	F	Adult	84.630	Positive
	B3	172	F	Sub-adult	89.364	Positive
	C3	177	F	Adult	86.900	Positive
	D3	178	M	Adult	87.484	Positive
	E3	180	F	Adult	89.364	Positive
	F3	181	M	Juvenile	46.563	Positive
	G3	182	F	Adult	87.160	Positive
	H3	184	F	Adult	83.528	Positive
	A4	185	M	Adult	88.197	Positive
	B4	186	M	Juvenile	89.040	Positive
	C4	187	F	Juvenile	89.040	Positive
	D4	188	F	Adult	88.457	Positive
	E4	189	M	Adult	87.808	Positive
	F4	190	M	Juvenile	89.948	Positive
	G4	191	F	Adult	87.289	Positive
	H4	192	F	Adult	89.494	Positive
	A5	193	F	Juvenile	86.706	Positive
Gariep GP	B5	194	M	Adult	89.235	Positive
	C5	196	M	Adult	87.289	Positive
	D5	198	F	Adult	88.716	Positive
	E5	200	F	Adult	88.327	Positive
	F5	201	F	Adult	88.262	Positive
	G5	202	M	Adult	87.549	Positive
	H5	203	M	Adult	80.091	Positive
	A6	206	M	Adult	87.030	Positive
	B6	207	M	Juvenile	88.586	Positive
	C6	209	F	Sub-adult	87.808	Positive
	E6	210	F	Sub-adult	89.040	Positive
	F6	212	F	Adult	89.300	Positive
	G6	213	M	Adult	86.965	Positive
	H6	218	F	Sub-adult	85.538	Positive
	A7	219	F	Juvenile	84.306	Positive
	B7	220	M	Adult	88.003	Positive
	C7	222	F	Adult	87.160	Positive
	D7	223	F	Adult	88.521	Positive
	F7	224	F	Adult	88.003	Positive

	G7	227	F	Adult	84.241	Positive
	H7	228	F	Adult	87.289	Positive
	A8	229	F	Juvenile	88.521	Positive
	B8	230	F	Adult	87.419	Positive
	C8	232	F	Adult	88.586	Positive
	D8	234	M	Juvenile	88.067	Positive
	E8	235	F	Adult	85.863	Positive
	F8	236	F	Adult	86.187	Positive
	G8	240	F	Sub-adult	88.392	Positive
	H8	243	F	Adult	85.733	Positive
	A9	249	M	Adult	86.122	Positive
Sandveld SAV	B9	267	M	Adult	80.674	Positive
	C9	269	M	Adult	87.613	Positive
	D9	270	M	Adult	88.327	Positive
	E9	272	F	Sub-adult	87.224	Positive
	F9	273	F	Sub-adult	87.354	Positive
	G9	274	F	Adult	88.003	Positive
	H9	275	F	Adult	84.760	Positive
	A10	276	M	Adult	86.057	Positive
	B10	277	M	Adult	84.436	Positive
	C10	278	M	Adult	88.781	Positive
	D10	280	M	Adult	88.911	Positive
	E10	281	M	Adult	85.149	Positive
	F10	282	M	Adult	86.122	Positive
	G10	283	F	Adult	86.770	Positive
	H10	284	M	Adult	85.863	Positive
	A11	287	F	Adult	85.344	Positive
	B11	288	M	Sub-adult	86.316	Positive
	C11	291	F	Adult	89.364	Positive
	D11	293	M	Sub-adult	73.346	Positive
	E11	294	F	Adult	88.975	Positive
	F11	295	F	Adult	88.716	Positive
	G11	296	F	Sub-adult	88.846	Positive
	H11	297	F	Adult	84.306	Positive
	A12	298	F	Adult	73.346	Positive
	C12	299	F	Adult	84.436	Positive
	D12	300	F	Adult	83.787	Positive
	E12	301	F	Adult	86.576	Positive
	F12	302	M	Adult	83.204	Positive
	G12	303	M	Adult	84.047	Positive
	H12	304	F	Adult	80.415	Positive

Plate 3	Well number	Lab ID	Sex	Age	Percentage inhibition	Result
Tussen-die-Riviere TDR	C1	1	M	Adult	89.552	Positive
	D1	7	F	Adult	90.665	Positive
	E1	11	F	Adult	90.066	Positive
	F1	15	M	Adult	85.612	Positive
	H1	16	F	Adult	72.937	Positive
	A2	17	F	Adult	80.303	Positive
	B2	18	F	Adult	91.778	Positive
	C2	19	M	Adult	92.635	Positive
	D2	20	F	Adult	91.607	Positive
	E2	21	F	Adult	76.706	Positive
	F2	22	F	Adult	91.436	Positive
	G2	25	F	Adult	91.350	Positive
	H2	33	F	Juvenile	81.073	Positive
Schoondal TD	A3	60	M	Adult	85.869	Positive
	B3	62	F	Sub-adult	89.381	Positive
	C3	65	F	Sub-adult	91.008	Positive
	D3	66	M	Adult	90.408	Positive
	E3	69	F	Adult	90.922	Positive
Grootkoppe EE	F3	72	F	Adult	89.894	Positive
	G3	73	F	Adult	91.522	Positive
	H3	74	M	Adult	89.552	Positive
	A4	75	M	Juvenile	91.093	Positive
	B4	76	F	Adult	88.781	Positive
	C4	77	F	Adult	91.778	Positive
	D4	85	F	Adult	91.693	Positive
	E4	86	M	Adult	84.328	Positive
	F4	87	F	Adult	91.008	Positive
	G4	91	F	Adult	69.854	Positive
	H4	92	F	Adult	91.008	Positive
	A5	97	M	Sub-adult	91.436	Positive
	B5	100	M	Adult	90.665	Positive
Copperfield JF	C5	111	M	Adult	91.864	Positive
	D5	114	M	Adult	90.408	Positive
	E5	115	M	Adult	85.098	Positive
	F5	117	F	Sub-adult	91.436	Positive
	G5	121	F	Adult	91.179	Positive
	H5	123	F	Adult	89.894	Positive
	A6	124	F	Adult	89.381	Positive
	B6	125	F	Adult	72.766	Positive
	C6	127	F	Adult	80.046	Positive
	E6	128	F	Juvenile	91.778	Positive
	F6	129	F	Adult	91.008	Positive
	G6	130	F	Juvenile	90.408	Positive
Maria Moroka MM	H6	139	M	Sub-adult	91.179	Positive
	A7	144	M	Adult	89.723	Positive
	B7	145	M	Adult	92.121	Positive
	C7	150	F	Adult	91.350	Positive
	D7	156	M	Adult	85.270	Positive
	F7	158	F	Adult	84.071	Positive

	G7	160	F	Adult	91.265	Positive
	H7	161	F	Adult	90.751	Positive
	A8	164	M	Sub-adult	88.182	Positive
	B8	165	F	Adult	90.151	Positive
	C8	166	F	Adult	89.637	Positive
	D8	167	F	Adult	91.522	Positive
	E8	168	M	Adult	85.698	Positive
	F8	169	M	Adult	91.350	Positive
	G8	171	F	Adult	82.101	Positive
	H8	174	F	Juvenile	89.894	Positive
	A9	176	F	Adult	29.004	Positive
	B9	179	F	Juvenile	90.151	Positive
	C9	183	F	Adult	84.670	Positive
Gariep GP	D9	195	F	Sub-adult	87.925	Positive
	E9	197	F	Sub-adult	88.010	Positive
	F9	204	M	Adult	92.292	Positive
	G9	205	M	Adult	90.494	Positive
	H9	214	M	Adult	89.723	Positive
	A10	231	M	Adult	79.446	Positive
	B10	233	M	Adult	91.950	Positive
	C10	238	F	Adult	81.416	Positive
	D10	239	F	Adult	91.778	Positive
	E10	241	F	Adult	92.721	Positive
	F10	242	M	Adult	92.121	Positive
	G10	247	M	Adult	89.466	Positive
	H10	250	M	Adult	91.265	Positive
	A11	251	F	Sub-adult	86.554	Positive
	B11	252	M	Sub-adult	91.693	Positive
	C11	253	F	Adult	87.496	Positive
	D11	255	F	Adult	91.950	Positive
	E11	256	M	Juvenile	92.292	Positive
	F11	260	F	Adult	92.977	Positive
	G11	262	F	Adult	90.751	Positive
Sandveld SAV	H11	268	M	Adult	91.607	Positive
	A12	271	F	Adult	65.829	Positive
	C12	279	M	Adult	86.983	Positive
	D12	285	F	Adult	89.295	Positive
	E12	286	M	Adult	89.894	Positive
	F12	289	F	Sub-adult	82.727	Positive
	G12	290	F	Adult	90.922	Positive
	H12	292	F	Adult	87.582	Positive

APPENDIX III: MELTING TEMPERATURES OF THE CONTROLS

LightCycler run	Melting temperatures (°C)			Difference between
	H2O	4408	WB5	WB5 and 4408
1 to 27	85.04	88.67	89.34	0.67
28 to 56	86.69	88.15	88.84	0.69
57 to 85	84.42	88.33	88.96	0.63
86 to 114	85.10	89.01	89.63	0.62
115 to 143	85.47	88.99	89.69	0.70
144 to 172	85.38	88.88	89.57	0.69
173 to 201	85.06	88.79	89.40	0.61
202 to 231	85.22	89.08	89.63	0.55
232 to 260	84.60	88.86	89.58	0.72
261 to 289	84.66	88.69	89.18	0.49
290 to 14-1	85.34	89.16	89.91	0.75
18-1 to 177-1	85.12	89.02	89.70	0.68
178-1 to 206-1	85.39	89.03	89.64	0.61
207-1 to 236-1	84.55	88.34	88.98	0.64
237-1 to 271-1	85.65	88.80	89.46	0.66
272-1 to 300-1	85.32	88.75	89.36	0.61
301-1 to 26-2	84.55	88.36	89.03	0.67
27-2 to 144-2	84.63	88.70	89.28	0.58
145-2 to 173-2	84.66	88.79	89.15	0.36
Test run 1	83.64	88.03	88.76	0.73
Test run 2	83.70	88.11	88.71	0.60
174-2 to 202-2	85.51	89.25	90.00	0.75
203-2 to 231-2	85.55	89.18	89.84	0.66
233-2 to 264-2	84.76	88.68	89.31	0.63
265-2 to 294-2	85.52	89.25	89.90	0.65
295-2 to 201-3	85.32	88.85	89.48	0.63
206-3 to 294-3	85.12	89.09	89.75	0.66
297-3 to 223-4	85.15	89.20	89.83	0.63
224-4 to 304-4	84.92	89.22	89.88	0.66
Foetal cornea rerun	85.11	88.02	89.52	1.50
Foetal spleen rerun	85.24	88.54	89.93	1.39
EDTA & Spleen rerun	84.71	88.20	89.67	1.47
Cornea rerun	84.91	88.41	89.98	1.57
Total	**2806.01**	**2928.43**	**2952.89**	**24.46**
Mean melting temperature	**85.03060606**	**88.74030303**	**89.48151515**	**0.741212121**
	85.03	**88.74**	**89.45**	**0.71**
Highest T_m	86.69	89.25	90.00	
Lowest T_m	83.64	88.02	88.71	
Standard deviation	**0.560925574**	**0.379806694**	**0.369678915**	

APPENDIX IV: REAL-TIME PCR RESULTS

Lab ID	Sample ID	Sex	Age	Samples			Foetal samples		
Tussen-die-Riviere				EDTA	Spleen	Cornea	Spleen	Cornea	Sex
WD	TDR			No	No – 1	No – 2	No – 3	No – 4	
1	1	M	Adult	+					
2	2	F	Sub-adult						
3	3	M	Adult						
4	4	F	Adult			+			
5	5	F	Adult			+			
6	6	F	Sub-adult						
7	7	F	Adult						
8	8	M	Adult			+			
9	9	F	Adult						
10	10	F	Adult						
11	11	F	Adult						
12	12	F	Sub-adult						
13	13	F	Sub-adult						
14	14	F	Sub-adult			+			
15	20	M	Adult						
16	21	F	Adult						
17	22	F	Adult						
18	23	F	Adult						
19	24	M	Adult						
20	25	F	Adult						
21	28	F	Adult						
22	29	F	Adult						
23	30	F	Adult				+		
24	31	F	Juvenile						
25	32	F	Adult						
26	33	F	Adult						
27	34	F	Adult				+		
28	35	F	Adult						
29	36	F	Adult						
30	37	F	Adult						
31	38	F	Adult				+		
32	39	F	Adult						
33	40	F	Juvenile						
34	41	F	Adult				+	+	
35	42	F	Adult					+	
36	43	F	Sub-adult						
37	44	F	Juvenile						
38	45	F	Adult			+			
39	46	F	Sub-adult						
40	47	M	Adult						
41	48	M	Adult						
42	49	F	Adult					+	
43	50	F	Adult				+	+	
Schoondal									
WD	TD								
44	101	M	Adult						
45	102	M	Adult						
46	103	M	Adult						
47	104	M	Adult						
48	105	M	Adult						
49	106	M	Adult						

Lab ID	Sample ID	Sex	Age	Samples			Foetal samples		
				EDTA	Spleen	Cornea	Spleen	Cornea	Sex
				No	No – 1	No – 2	No – 3	No – 4	
50	107	M	Adult						
51	108	M	Adult						
52	109	M	Adult						
53	110	M	Adult						
54	111	M	Adult						
55	112	M	Adult	+					
56	113	M	Adult	+					
57	114	M	Adult						
58	115	M	Adult						
59	116	M	Adult						
60	117	M	Adult						
61	118	M	Adult						
62	119	F	Sub-adult						
63	120	M	Sub-adult						
64	121	M	Sub-adult						
65	122	F	Sub-adult						
66	123	M	Adult						
67	124	F	Adult						
68	125	F	Adult						
69	126	F	Adult						
70	127	M	Juvenile						
Grootkoppe									
WD	**EE**								
71	201	M	Juvenile						
72	202	F	Adult						
73	203	F	Adult						
74	204	M	Adult						
75	205	M	Juvenile	+					
76	206	F	Adult						
77	207	F	Adult						
78	208	F	Adult						
79	209	F	Adult						
80	210	M	Juvenile						
81	211	F	Juvenile						
82	212	F	Juvenile						
83	213	F	Adult						
84	214	F	Juvenile						
85	215	F	Adult						
86	216	M	Adult	+					
87	217	F	Adult						
88	218	M	Juvenile						
89	219	F	Adult						
90	220	M	Juvenile	+					
91	221	F	Adult						
92	222	F	Adult						
93	223	F	Adult						
94	224	F	Adult						
95	225	F	Juvenile						
96	226	M	Adult						
97	227	M	Sub-adult	+					
98	228	M	Adult						
99	229	M	Adult						
100	230	M	Adult						
101	231	M	Adult						
102	232	M	Adult						

Lab ID	Sample ID	Sex	Age	Samples			Foetal samples		
				EDTA	Spleen	Cornea	Spleen	Cornea	Sex
				No	No – 1	No – 2	No – 3	No – 4	
Copperfield									
WD	**JF**								
103	301	F	Sub-adult						
104	302	F	Juvenile						
105	303	M	Adult						
106	304	M	Adult						
107	305	M	Adult						
108	306	M	Adult						
109	307	M	Adult						
110	308	M	Adult						
111	309	M	Adult						
112	310	M	Adult						
113	311	M	Adult						
114	312	M	Adult						
115	313	M	Adult						
116	314	M	Adult						
117	315	F	Sub-adult						
118	316	F	Adult						
119	317	F	Juvenile						
120	318	F	Adult						
121	319	F	Adult						
122	320	F	Adult						
123	321	F	Adult						
124	322	F	Adult						
125	323	F	Adult						
126	324	F	Adult						
127	325	F	Adult						
128	326	F	Juvenile						
129	327	F	Adult						
130	328	F	Juvenile						
131	329	F	Adult						
132	330	M	Juvenile						
133	331	F	Juvenile						
134	332	M	Juvenile						
Maria Moroka									
WD	**MM**								
135	401	M	Adult						
136	402	M	Adult						
137	403	M	Sub-adult						
138	404	M	Adult						
139	405	M	Sub-adult						
140	406	M	Adult						
141	407	M	Adult						
142	408	F	Adult			+			
143	409	M	Sub-adult						
144	410	M	Adult						
145	411	M	Adult			+			
146	412	F	Adult						
147	413	M	Adult			+			
148	414	M	Adult						
149	415	M	Adult						
150	416	F	Adult						
151	417	M	Adult						
152	418	F	Adult						
153	419	M	Adult						
154	420	M	Adult			+			
155	421	F	Adult						

Lab ID	Sample ID	Sex	Age	Samples			Foetal samples		
				EDTA	Spleen	Cornea	Spleen	Cornea	Sex
No				No	No – 1	No – 2	No – 3	No – 4	
156	422	M	Adult						
157	423	F	Adult						
158	424	F	Adult						
159	425	M	Adult						
160	426	F	Adult						
161	427	F	Adult						
162	428	M	Adult						
163	429	F	Adult						
164	430	M	Sub-adult			+			
165	431	F	Adult						
166	432	F	Adult						
167	433	F	Adult			+			
168	434	M	Adult			+			
169	435	M	Adult			+			
170	436	M	Adult			+			
171	437	F	Adult						
172	438	F	Sub-adult						
173	439	F	Adult			+			
174	440	F	Juvenile						
175	441	F	Adult						
176	442	F	Adult						
177	443	F	Adult						
178	444	M	Adult						
179	445	F	Juvenile						
180	451	F	Adult				+	+	
181	452	M	Juvenile						
182	453	F	Adult				+		
183	454	F	Adult					+	
184	455	F	Adult					+	
185	457	M	Adult						
186	458	M	Juvenile						
187	459	F	Juvenile		+	+			
188	460	F	Adult				+	+	
189	461	M	Adult						
190	462	M	Juvenile						
191	463	F	Adult					+	
192	464	F	Adult					+	
193	465	F	Juvenile						
Gariep									
WD	GP								
194	501	M	Adult						
195	502	F	Sub-adult						
196	503	M	Adult						
197	504	F	Sub-adult						
198	505	F	Adult				+		F
199	506	M	Adult						
200	507	F	Adult				+		M
201	508	F	Adult				+		M
202	509	M	Adult						
203	510	M	Adult						
204	511	M	Adult						
205	512	M	Adult						
206	513	M	Adult				+		F
207	514	M	Juvenile						
208	515	F	Adult						
209	516	F	Sub-adult		+				
210	517	F	Sub-adult						

211	518	F	Adult						
Lab ID	**Sample ID**	**Sex**	**Age**	**Samples**			**Foetal samples**		
				EDTA	**Spleen**	**Cornea**	**Spleen**	**Cornea**	**Sex**
				No	**No – 1**	**No – 2**	**No – 3**	**No – 4**	
212	519	F	Adult			+	+		F
213	520	M	Adult		+	+			
214	521	M	Adult						
215	522	F	Adult				+		F
216	523	M	Adult						
217	524	F	Adult				+		F
218	525	F	Sub-adult						
219	526	F	Juvenile			+			
220	527	M	Adult						
221	528	F	Adult				+		M
222	529	F	Adult				+	+	F
223	530	F	Adult				+		M
224	531	F	Adult				+		M
225	532	F	Juvenile						
226	533	M	Adult						
227	534	F	Adult			+	+		F
228	535	F	Adult			+	+		F
229	536	F	Juvenile						
230	537	F	Adult						
231	538	M	Adult						
232	539	F	Adult		+				
233	540	M	Adult	+					
234	541	M	Juvenile			+			
235	542	F	Adult				+		M
236	543	F	Adult			+	+		F
237	544	F	Adult				+		F
238	545	F	Adult			+			
239	546	F	Adult				+		F
240	547	F	Sub-adult						
241	548	F	Adult				+		M
242	549	M	Adult						
243	550	F	Adult						F
244	551	F	Adult	+			+		F
245	552	F	Sub-adult						
246	553	M	Sub-adult						
247	554	M	Adult						
248	555	F	Adult						
249	556	M	Adult						
250	557	M	Adult						
251	558	F	Sub-adult						
252	559	M	Sub-adult						
253	560	F	Adult				+		F
254	561	F	Adult				+		M
255	562	F	Adult				+		F
256	563	M	Juvenile						
257	564	F	Juvenile						
258	565	F	Adult						
259	566	F	Adult				+		F
260	567	F	Adult				+		F
261	568	F	Adult				+		F
262	569	F	Adult						
263	570	M	Adult						
264	571	F	Adult				+		F
265	572	F	Adult				+		M
266	Thaba Manzi	F	Juvenile						

Lab ID	Sample ID	Sex	Age	Samples			Foetal samples		
				EDTA	Spleen	Cornea	Spleen	Cornea	Sex
				No	No – 1	No – 2	No – 3	No – 4	
Sandveld									
WD	SAV								
267	601	M	Adult						
268	602	M	Adult		+				
269	603	M	Adult						
270	604	M	Adult						
271	605	F	Adult		+				
272	606	F	Sub-adult		+				
273	607	F	Sub-adult		+				
274	608	F	Adult		+				
275	609	F	Adult		+		+	+	M
276	610	M	Adult						
277	611	M	Adult						
278	612	M	Adult						
279	613	M	Adult		+				
280	614	M	Adult						
281	615	M	Adult						
282	616	M	Adult		+				
283	617	F	Adult						
284	618	M	Adult			+			
285	619	F	Adult						
286	620	M	Adult		+				
287	621	F	Adult				+	+	M
288	622	M	Sub-adult						
289	623	F	Sub-adult						
290	624	F	Adult				+		F
291	625	F	Adult						
292	626	F	Adult		+				
293	627	M	Sub-adult						
294	628	F	Adult						F
295	629	F	Adult						
296	630	F	Sub-adult						
297	631	F	Adult				+	+	M
298	632	F	Adult			+	+	+	M
299	633	F	Adult						
300	634	F	Adult		+	+			
301	635	F	Adult						
302	636	M	Adult						
303	637	M	Adult						
304	638	F	Adult						M

Foetal samples No samples taken Negative result