CHAPTER SIX:

DETECTION AND CHARACTERIZATION OF PAPILLOMAVIRUS SKIN LESIONS OF GIRAFFE (*Giraffa camelopardalis*, Linnaeus, 1758) AND SABLE ANTELOPE (*Hippotragus niger*, Harris, 1838) IN SOUTH AFRICA

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ABSTRACT

Unsightly horny outgrowths in the skin of the giraffe (*Giraffa camelopardalis*) have been reported in the Kruger National Park, Mpumalanga and two giraffes were euthanased due to this condition. A sable antelope (*Hippotragus niger*), on a game farm in the Kimberley district, Northern Cape, exhibited lameness due to a cutaneous growth, which was removed surgically.

Papillomavirus was detected electron microscopically in cutaneous fibropapillomas in these giraffes and sable antelope. The virus particles measured 45 nm in diameter. Histopathologically, the lesions showed features similar to those of equine sarcoid as well as positive immunoperoxidase-staining of tissue sections for papillomavirus antigen. Polymerase chain reaction demonstrated the presence of BPV DNA. Bovine papillomavirus-1 was detected by real-time PCR in the sable and giraffe, and cloning and sequencing of the PCR–product showed the highest similarity (99%) to BPV-1 found in Cape mountain zebras. All sequences from the giraffe and sable were closely related (97%) to the BPV-1 subtype IV, detected in equine sarcoid.

In a similar lesion in a second giraffe histopathologically malignant pleomorphism was detected indicating the end-point of papilloma infection. Neither virus particles nor positively staining papillomavirus antigen could be demonstrated but papillomavirus DNA was detected by real-time PCR which corresponded to BPV-1 and BPV-2.

Keywords: Cutaneous fibropapillomas, giraffe (*Giraffa camelopardalis*), malignant pleomorphism, sable antelope (*Hippotragus niger*), papillomavirus.

6.1 INTRODUCTION

Papillomaviruses are classified in the family *Papillomaviridae* (Bernard 2006). This large family of animal and human viruses generally infects epithelial cells causing hyperproliferative lesions. It has also oncogenic potential (Lambert *et al*. 2006), infecting cutaneous and mucous epithelia in a variety of hosts through cuts or abrasions, and induces the formation of papillomas or warts by targeting the keratinocyte, the viral growth accompanying the steady maturation of the cell to the surface (Stanley 2006). These tumours are generally benign and self-limiting, and spontaneously regress with the animal recovering completely, but occasionally benign tumours may persist and become malignant by progressing to squamous cell carcinoma (Campo 2006).

Bovine papillomavirus (BPV) induces exophytic papillomas of cutaneous or mucosal epithelia in cattle. The papillomas are benign tumours which generally regress uneventfully; however, they do occasionally persist and provide the focus for malignant transformation to squamous cell carcinomas, particularly in the presence of environmental co-factors. This has been experimentally demonstrated in cancer of the urinary bladder and upper alimentary tract in cattle feeding on bracken fern (*Pteridium aquilinum*) (Wosiacki *et al*. 2005). In equines BPV cause a locally invasive, fibroblastic skin tumour of horses, donkeys, mules (Nasir & Reid 2006) as well as in the zebra. These tumours appear as different clinical entities and can be classified into six clinical types (Knottenbelt 2005). This cross-species infection of Equidae by BPV-1 and -2 is the only record until recently of a papillomavirus cross-infecting species barriers, the resulting tumour is being known as a sarcoid (Nasir *et al*. 2007). Bovine papilloma virus has been demonstrated in lesions in the waterbuffalo (*Bubalis bubalis*) (Silvestre *et al*. 2009), and bison (*Bison bonasus*) (Literák *et al*. 2006).

Apart from BPV-1 and -2, papillomaviruses are strictly species-specific and only the natural host is infected; even under experimental conditions papillomaviruses do not infect any host other than the natural one. Lesions usually attributed to papillomavirus infection have been most extensively studied in cattle (Jarret *et al*. 1985; Anderson *et al*. 1997; Campo 2002) and horses (Angelos *et al*. 1991; Otten *et al.* 1993; Reid *et al*. 1994; Carr *et al*. 2001; Chambers *et al*. 2003) but at least 50 mammalian species have been confirmed as being infected by species-specific papillomaviruses (Sundberg *et al*. 2001). Papillomaviruses appear to be widespread and have been found in a large number of vertebrate species and are assumed to have evolved alongside their hosts (Bernard 1994; Antonssun & McMillan 2006). Virtually all mammalian species are hosts for one or more paplillomavirus (Sundberg *et al.* 2001).

An adult female giraffe (*Giraffa camelopardalis*) (Giraffe 1) with extensive lesions in the skin of the dorsal neck and back was observed near the Shingwedzi Restcamp in the northern part of the Kruger National Park (KNP), South Africa (**Figure 23**). She was in a group of six animals, one of which was a bull. The other animals in the group showed no lesions. This area is a natural habitat for giraffe as they occur in a variety of dry savanna ranging from scrub to woodland (Smithers, 1983). Approximately one year later a second affected giraffe (Giraffe 2), was observed in the vicinity of the Skukuza Restcamp and the Kruger Gate in the southern part of the KNP (**Figure 24**). This was also an adult female and part of a mixed group, regarding sex and age, of five animals. She was the only one in the group exhibiting wart-like lesions which were especially prominent in the skin of her head and neck, but spread to the rest of her body skin had occurred.

A group of 30 sable antelope (*Hippotragus niger*) was kept as a breeding herd on a game farm in the Kimberley district, Northern Cape Province, South Africa. This arid area of South Africa is not the natural habitat of sable as they are a savanna woodland species, but game are at times translocated to private land and reserves situated out of their normal range. One cow was lame in the right hind leg and a wart-like lesion of 60 mm x 60 mm was present in the skin of the lateral aspect of the distal part of the second phalanx proximal to the right hind hoof. After surgical removal growth recurred at the original site and other well-defined wart-like lesions were also noticed in the skin of the right shoulder and lip.

The purpose of this study was to determine electron microscopically if BPV was present in the lesions of these animals and, if so, to detect and distinguish between BPV-1 and -2 DNA using real-time PCR.

6.2 MATERIALS AND METHODS

6.2.1 Sample collection

Giraffe 1 was shot in May 2007 in the vicinity of the Shingwedzi Restcamp KNP at the geographic position of 23˚97'42"S and 31˚42'58"E. Samples of the lesions were collected for PCR by excising pieces of tissue 10 mm x 3 mm x 3 mm in size. These were stored in sterile tubes at 4 °C until analysis. Papilloma tissue samples for histopathological examination were stored in duplicate in a 10% dilution of formaldehyde. The second giraffe (Giraffe 2) was shot near Skukuza Restcamp at the geographical position of 24˚59'16"S and 31˚34'32"E in October 2008 and samples were collected as mentioned above. The sable antelope was anaesthetized in the Kimberley district at the

geographic position of 28˚44'0" S and of 24˚46'0"E and the lesion on the pastern was surgically removed in an effort to relieve the lameness. Samples of the lesion were taken as described above.

6.2.2 Electron microscopy

Formalin fixed skin samples from both giraffes were prepared for transmission electron microscopy (TEM) according to standard procedures. Ultra thin sections were stained with uranyl acetate and lead citrate, and examined in a Philips CM 10 transmission electron microscope operated at 80 kV.

An unfixed skin sample of the sable cow was prepared for TEM by grinding the tissue in a mortar with a pestle in a small volume of sterile water and the mixture was centrifuged at 13 000 rpm for 45 minutes. The resultant pellet was re-suspended in water, stained with 3% phosphotungstic acid and a drop of the suspension placed onto a formvar and carbon coated grid for examination. A relevant area was also retrieved from the histological wax block (6.2.3), and was treated with 1% OsO_4 in Xylene and embedded in an epoxy resin.

6.2.3 Histopathology

The formalin-fixed samples from both giraffes and the sable were dehydrated and paraffin wax-embedded for routine histological processing. All tumour sections were stained with haematoxylin and eosin for light microscopy as well as with immunoperoxidase for immunohistochemical evaluation of bovine papillomavirus using the avidin-biotin technique (Haines & Chelack 1991). A polyclonal rabbit anti-papillomavirus antibody known to react with the L1 capsid proteins of most known papillomaviruses was used. Immunohistochemical staining using the avidin-biotin complex detection system was done on formalin-fixed, paraffin wax-embedded sections of the lesions. The antibody used was against chemically disrupted BPV-1. A section of a bovine fibropapilloma was used as a positive control.

6.2.4 DNA extraction

DNA was extracted from 25 mg of lesion tissues from each animal using the QIAamp®DNA extraction kit (Southern Cross Biotechnologies) according to the manufacturer's instructions. Extracted DNA was eluted in 100 µl elution buffer and stored at 4 °C until further analysis.

6.2.5 Real-time PCR

The hybridization probe real-time PCR assay, as described in Chapter 2 was used for the detection and differentiation of BPV-1 and -2 DNA in the giraffe (1 & 2) and sable lesions.

The PCR amplification mixture and reaction conditions were as described in Chapter 2. Fluorescence was measured at 640 and 705 nm and the results were analyzed with the Roche LightCycler® Software v4.0. Specimens, from positive sarcoid zebra (BPV-1 and -2) were included as positive controls.

6.2.6 Cloning and sequence analysis

Conventional PCR was used for the amplification of a 637 bp DNA fragment of a region of the E5 ORF of both BPV-1 and BPV-2. The primers, PCR amplification mixture and reaction conditions were as described in Chapter 2. However, a nested PCR step was included using 1 µl of the first round amplicons as PCR template in Giraffe 1 and the sable. The amplicons obtained were purified, cloned into the pGEM®-T easy vector (Promega pGEM–T Easy Vector System, Promega, Madison, USA), and transformed into competent JM 109 *E. coli* cells (Promega, Madison, USA). Recombinant plasmid DNA was isolated, directly sequenced using the ABI BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) and analyzed on an ABI 3100 sequencer at Inqaba Biotec (Pretoria, South Africa). Sequencing data were assembled and edited with the GAP4 program of the Staden package (version 1.6.0 for Windows) (Staden *et al.* 2000) and aligned with published sequences of related genera using ClustalX (version 1.81 for Windows). A BLAST search was performed using the Blastn algorithm. A phylogenetic tree was constructed using neighbour-joining (Saitou & Nei 1987) in combination with the bootstrap method (Felsenstein 1985) (1 000 replicates/tree for distance methods). Human papillomavirus type 16 (K02718) was used as an outgroup.

6.3 RESULTS

The extensive skin lesions on the head, neck and back of the two adult female giraffes (Giraffe 1 and 2) in the Kruger National Park can clearly be seen in **Figures 23** and **24**.

Figure 23 Multiple papillomas in the skin of Giraffe 1 euthanased in the vicinity of Shingwedzi Restcamp.

Figure 24 Giraffe 2 euthanased in the vicinity of Skukuza Restcamp. Note the presence of multiple nodular fibropapillomatous lesions in the skin of her neck, head and ears.

6.3.1 Electron microscopy

Negatively-stained spherical papillomavirus particles with a diameter of 52 nm and distinct capsomeres were present in the unfixed skin sample of the sable cow (**Figure 25**). The nuclei of the *stratum granulosum* of both the sable cow and Giraffe 1 contained numerous randomly scattered papillomavirus particles measuring 45 nm in diameter (**Figures 26 & 27**). The nuclei of infected cells displayed irregular clumps of condensed chromatin. No virus was demonstrated in samples obtained from Giraffe 2.

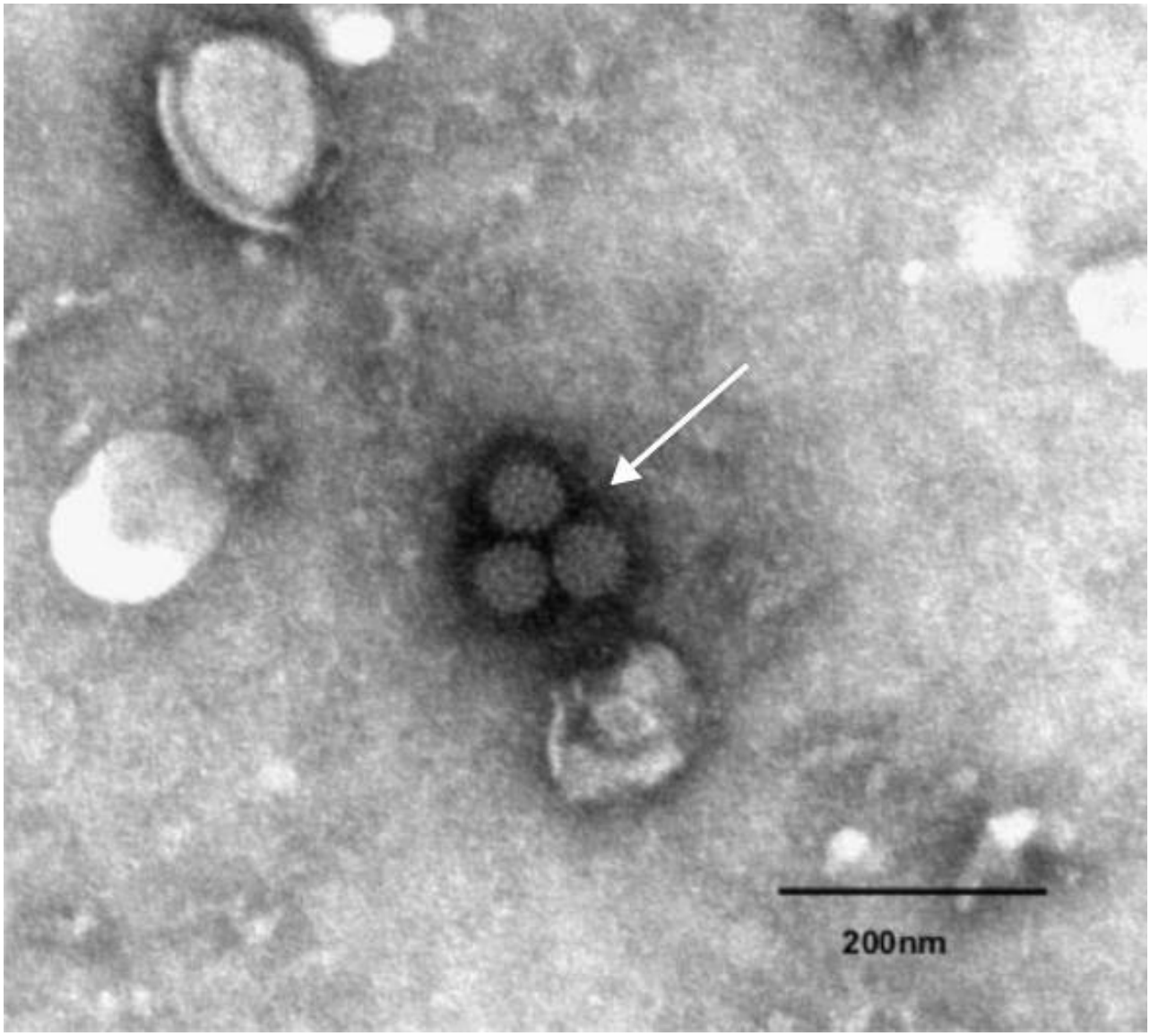

Figure 25 Negatively-stained intranuclear papillomavirus particles in an unfixed sample from the skin of the sable cow.

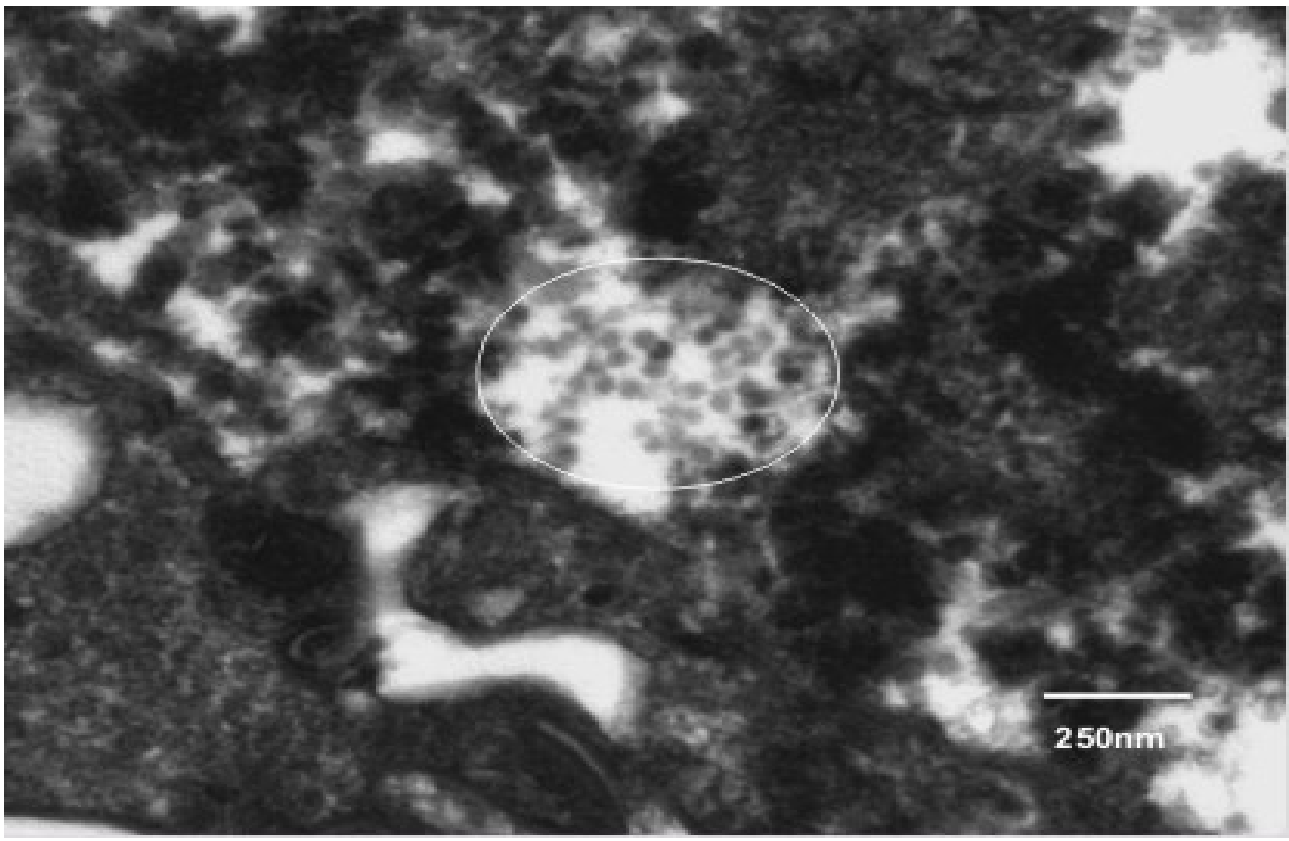

Figure 26 Intranuclear papillomavirus (encircled) particles stained positively with uranil-acetate in ultra thin sections of wax-retrieved epoxy sections of the sable cow skin.

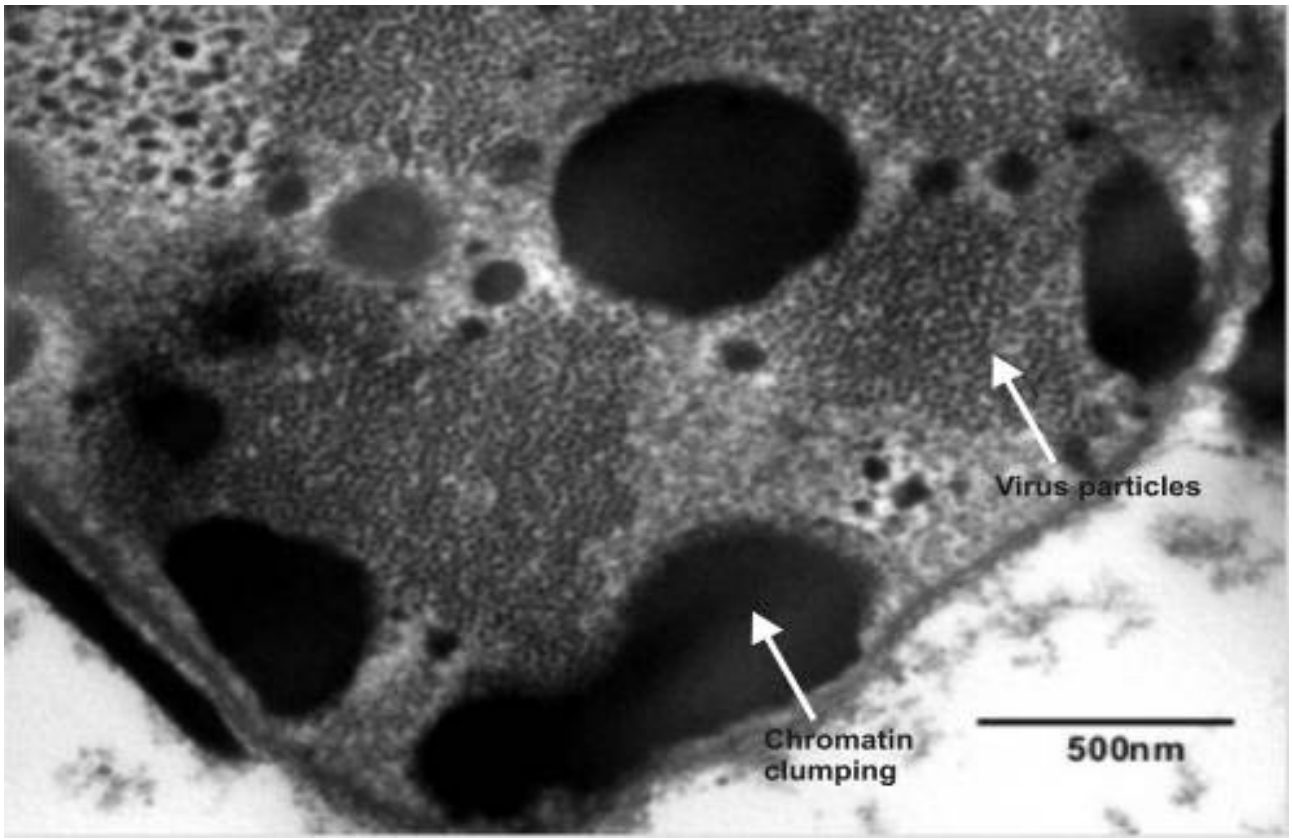

Figure 27 Numerous intranuclear papillomavirus particles in sections of formalin-fixed skin of Giraffe 1. Note nuclear chromatin clumping.

6.3.2 Histopathology

The skin lesions of Giraffe 1 were fibropapillomatous in nature and those of the sable resembled equine verrucose sarcoid. Small nuclei in the *stratum granulosum* of both animals stained positively for papillomavirus antigen (using the avidin-biotin complex detection system (Haines & Chelack 1991) (**Figure 28**). The lesions of Giraffe 2 were more sarcoid-like, expansile and infiltrative in the dermis and in some places showed evidence of malignancy. Scattered, large fibroblasts with bizarre large nuclei, some of which appeared multinucleated, occurred throughout the dermal tumour tissue (**Figure 29**). Immunohistochemical staining of sections from Giraffe 2 did not elucidate papillomavirus antigen.

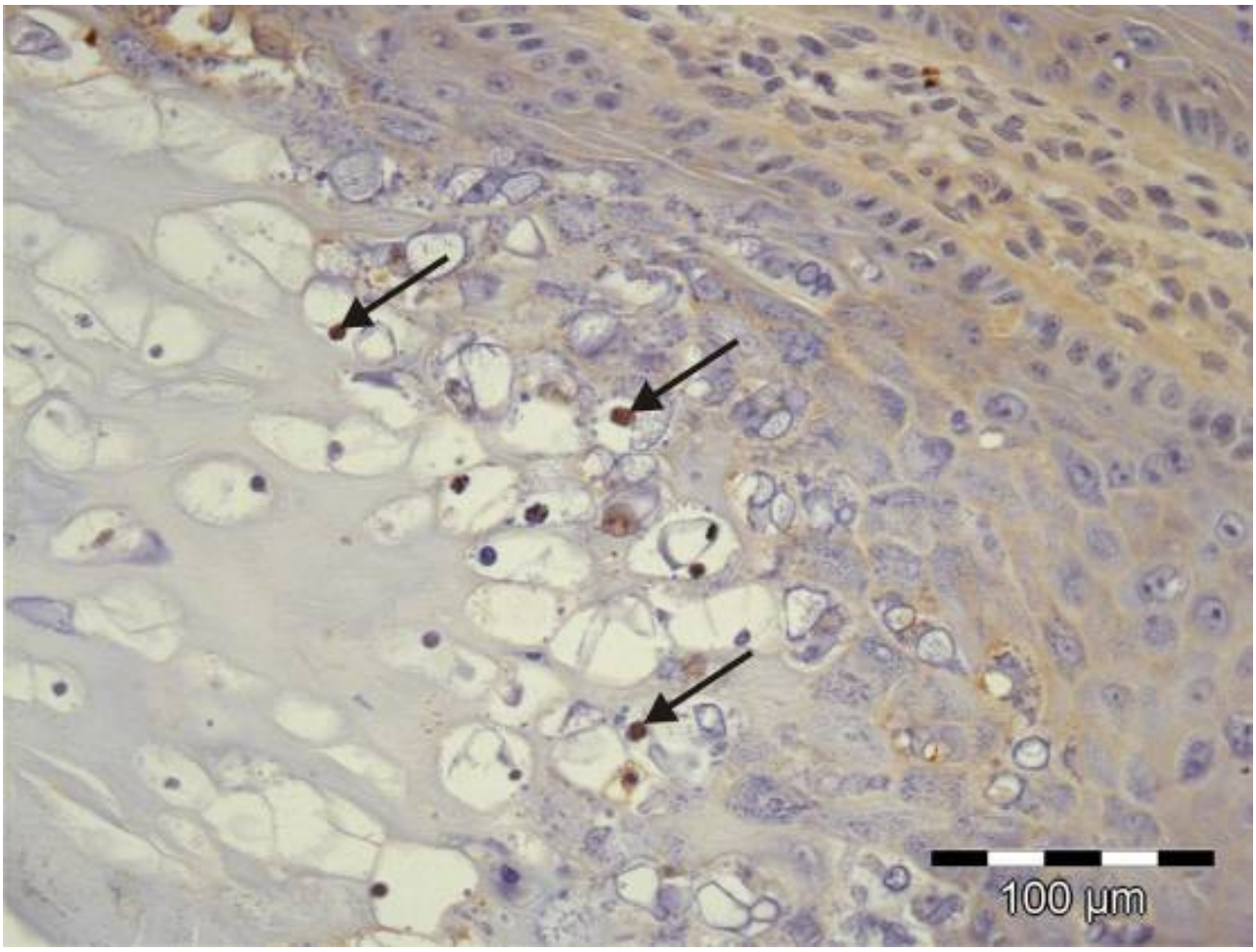

Figure 28 Lesion from sable showing occasional papillomavirus-positive *stratum granulosum* nuclei on immunohistochemistry stained with immunoperoxidase stain.

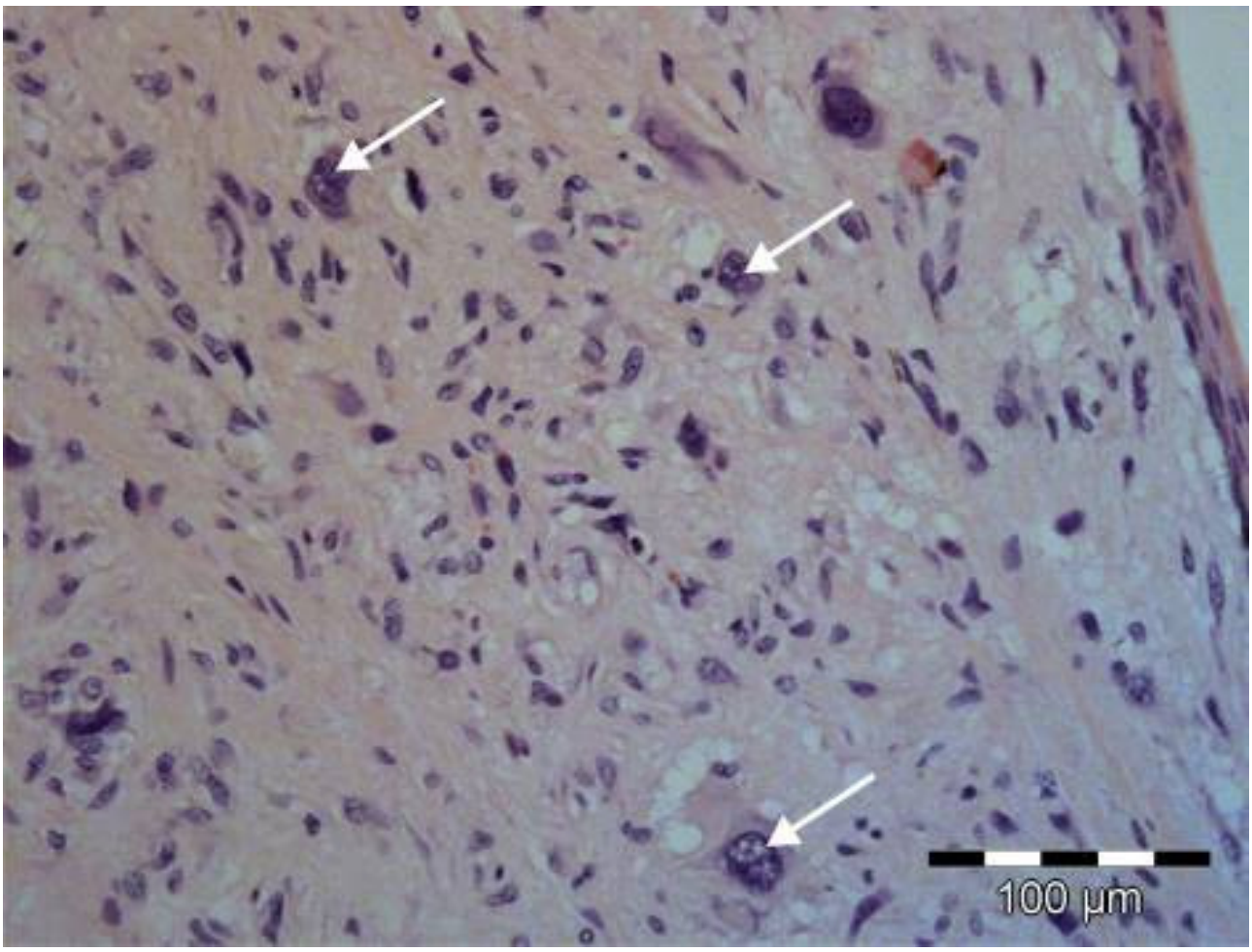

Figure 29 Giraffe 2: subepidermal sarcoid-like lesion stained with haematoxylin and eosin showing multifocal bizarre anaplastic fibroblasts (arrows).

6.3.3 Real-time PCR

The primers used in the real-time PCR assay amplified either a 244 bp or 247 bp region of the E5 ORF of BPV-1 and -2, respectively. Two separate hybridization probes sets were used in a multiplex format for the specific detection of BPV-1 and -2 DNA. For the detection of BPV-1 DNA, an increase in fluorescence is expected at 640 nm as well as two BPV-1-specific melting peaks at 62.90 ±1.24 °C and 68.17 ±0.71 °C. Similarly, for the detection of BPV-2 DNA, an increase in fluorescence at 705 nm is expected as well as two BPV-2-specific peaks at 58.86 ±0.60 °C and 64.06 ±0.59 °C.

Bovine papillomavirus 1-positive material from sarcoids of Cape mountain zebras induced a graph with melting peaks showing a similar configuration as those of material from Giraffe 1 and the sable. Giraffe 2 demonstrated fluorescence at 58 °C as well as 68 °C at 640 nm (**Figure 30**) indicating both BPV-1 and BPV-2.

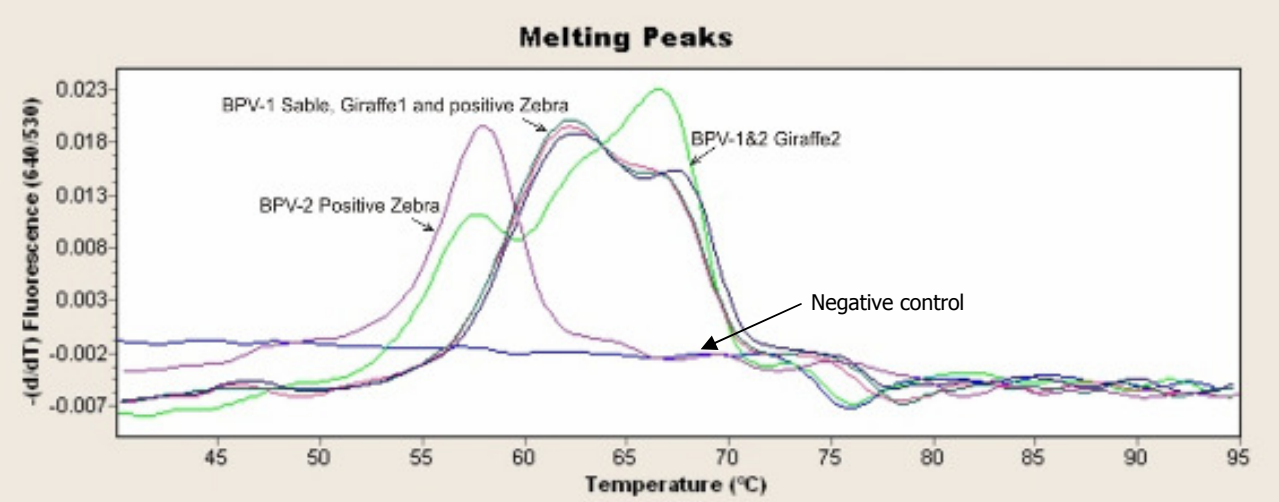

Figure 30 Real-time PCR results of the sable and Giraffe 1 demonstrating BPV-1 DNA, and showing an increase in fluorescence at 62.90±1.24 °C in both. The positive BPV-1 control zebra shows the same pattern while the control zebra for BPV-2 reveals an increased fluorescence at 58.48±0.61 °C. Giraffe 2 demonstrates a different graph fluorescing at 58 °C as well as 68 °C.

6.3.4 Cloning and sequencing

Fragments, 637 bp in size, of the E5 ORF of the sable and Giraffe 1 samples were amplified, cloned and sequenced to confirm that the amplicons obtained were indeed related to BPV. Sequences were edited and truncated to a length of 478 bp. Six [K10 (HM446170); K1 (HM446171); K3 (HM446172); K7 (HM446173); K5 (HM446174); K1-1 (HM446174)] of the nine Giraffe 1 clones had identical sequences over the 478 bp region and a BLAST search revealed that these sequences were identical to those of the BPV-1 E2 and E5 protein encoding genes previously reported in Chapter 2 to occur in Cape mountain zebras (FJ648519 to FJ648528). The other three clones [K4 (HM446168); K27 (HM446169); K2 (HM446176)] were not identical to each other, but the BLAST search showed the highest similarity (~99%) with the BPV-1 E2 and E5 protein encoding genes found in Cape mountain zebras (FJ648519 to FJ648528). Only two sequences were obtained from sable clones Sable 4 (HM 446166) and Sable 2 (HM 44167) and they showed two nucleotide differences within the 478 bp fragment. Again, the BLAST search showed the highest similarity (~99%) with the BPV-1 E2 and E5 protein encoding genes found in Cape mountain zebras. All

sequences obtained from Giraffe 1 and sable samples were closely related (~97%) to the BPV-1 subtype IV E5 protein encoding gene (accession number AY232260) detected in equine sarcoid (Chambers *et al*. 2003). A phylogenetic tree was constructed and the results were concurrent with the BLAST results obtained (**Figure 31**). In the case of Giraffe 2, no good quality sequence data could be obtained and the DNA could only be detected by real-time PCR.

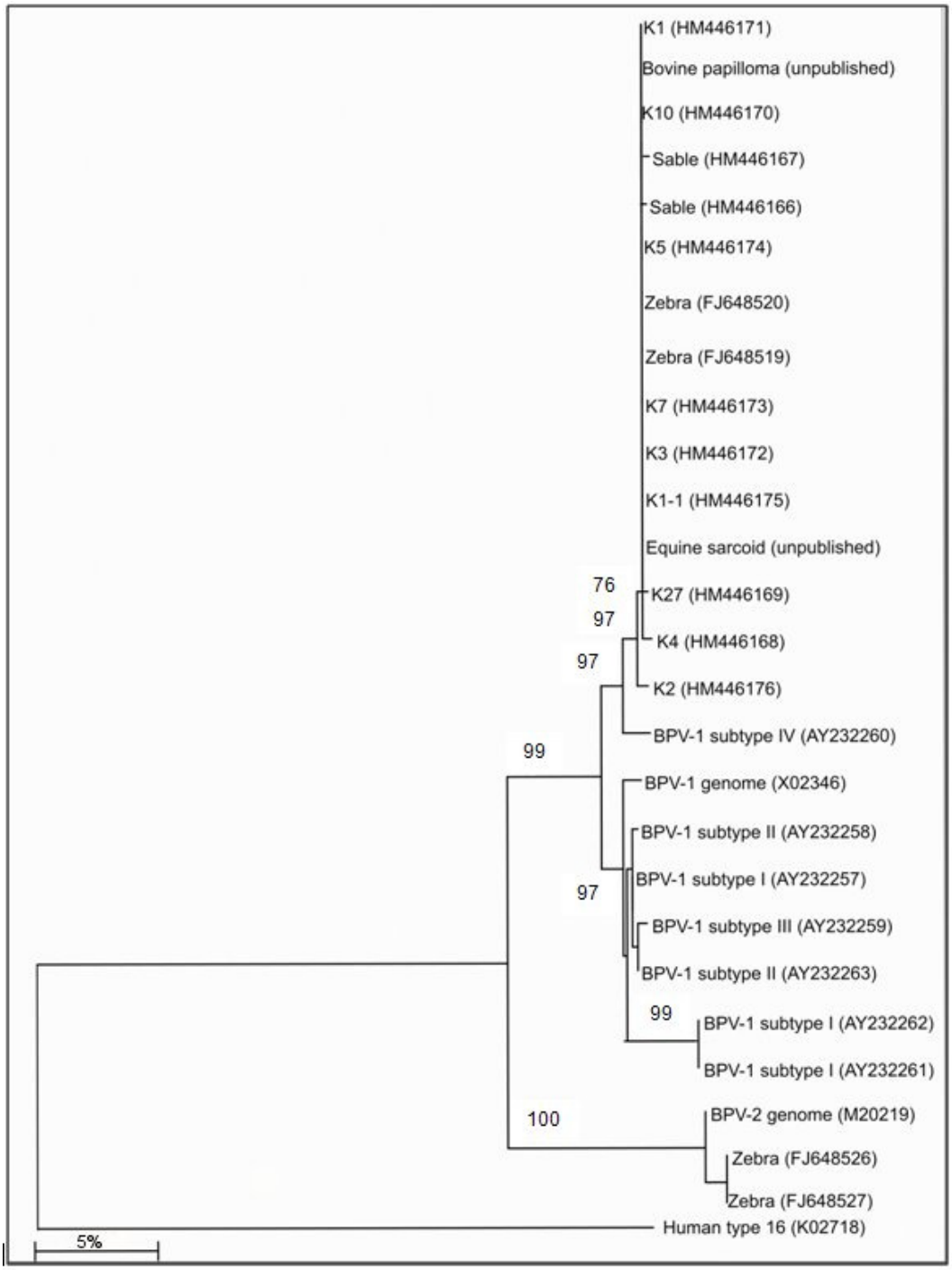

Figure 31 Results of the neighbour-joining tree of BPV E2E5 encoding gene showing the phylogenetic relationship of sequences compared to sequences of BPV-1 and BPV-2 obtained from GenBank.

6.4 DISCUSSION

Numerous descriptions of epithelial growths in the skin of many species of mammals have been reported (Karstad & Kaminjolo 1978; Uzal *et al*. 2000; Sundberg *et al*. 2000; 2001; Schulmann *et al*. 2001; 2003; Literák *et al*. 2006; Silvestre *et al.* 2009). Tustin (1978) reported epithelial growths in the giraffe in the Kruger National Park. They are characterized by a conspicuous amount of fibrous connective tissue at the base which forms cores upon which the neoplastic epithelial cells are massed, and are commonly associated with infection with a papillomavirus. This study documents the presence of BPV and/or DNA in such growths in the skin of the giraffe and a sable antelope.

Papillomatosis is a specific infectious disease in the species of animal in which it naturally occurs. The lesions are regarded as hyperplastic to a form of benign neoplasia as they do not metastasize and kill the host (Lancaster & Olson 1982). Although papillomavirus DNA is consistently found in the sarcoid lesions of the horse (Amtmann *et al*. 1980; Lancaster 1981) and have been found in the zebra in this study, papillomavirus particles have not been demonstrated, and the disease is therefore considered to be a non-productive infection in which the viral DNA exists episomally. The associated papillomavirus is consistently most closely related to BPV. Most fibropapillomas (sarcoids) in horses contain identifiable BPV DNA of either type 1 or 2 (Trendfield *et al.* 1985; Angelos *et al*. 1991; Otten *et al.* 1993; Reid *et al*. 1994; Carr *et al.* 2001; Chambers *et al.* 2003; Yuan *et al.* 2007). The E5 ORF of the papillomavirus identified in sarcoids of donkeys is very similar to BPV-1 and it has been proposed that it is a subtype of BPV-1 (Reid *et al.* 1994). In the present study the papillomavirus particles that we demonstrated in the wart-like lesions of the sable antelope and Giraffe 1 are considered to be closely related to BPV-1.

In this study, the virus particles demonstrated ultrastructurally in the sable and Giraffe 1, exhibited virus particles of 52 and 45 nm in diameter, with the typical nonenveloped isosahedral structure which forms paracrystalline arrays in the nucleus of infected cells. Infected nuclei display numerous irregular clumps of condensed chromatin and correspond to the description by Doane and Anderson (1987) for papillomavirus. The presence of the virus is further confirmed by the histological picture resembling equine sarcoid and staining histochemically positive for papillomavirus antigen.

Electron microscopically, virus particles have also been demonstrated in skin papillomas in an impala (*Aepyceros melampus*) and a giraffe (*Giraffa camelopardalis*) in Kenya by Karstad and Kaminjolo (1978) but sequencing was not done. In the water buffalo (*Bubalus bubalus*) (Silvestre *et al.* 2009) and the bison (*Bison bonasus*) (Literák *et al*. 2006) virus has also been demonstrated electron microscopically, and following its sequencing it was claimed to be homologous to the long control region of BPV-1 (prototype sequence, accession number X02346) in the water buffalo (Silvestre *et al*. 2009) while the DNA sequence of 413 bp amplicon derived from the European bison compared to the concensus sequence of BPV-2 (GenBank accession number AY300818) (Literák *et al*. 2006). The virus was also demonstrated ultrastructurally in both species of animals (Literák *et al*. 2006, Silvestre *et al.* 2009).

The real-time PCR method demonstrated the presence of BPV-1 in the sable and Giraffe 1 and was confirmed by cloning and sequencing. In the second giraffe, no good quality sequences could be obtained, although, the real-time PCR demonstrated the presence of both BPV-1 and -2. This can be attributed to the highly sensitive nature of the real-time PCR assay used, which is capable of demonstrating the virus of less than 1.5 gene copies as described in Chapter 2. Another reason why the virus could not be demonstrated was due to the fact that the papilloma had undergone a degree of cellular transformation, with more pleomorphic fibroblasts being present than in Giraffe 1. Once the papilloma has undergone malignant transformation to carcinoma, the structural integrity of the virus is lost and the virus or viral antigen is no longer present (Lancaster & Olson 1982).

In the phylogenetic tree (**Figure 31**), the clones obtained from the giraffe showed 99-100% similarity with the BPV DNA obtained from sable and the bovine papilloma and equine sarcoid that were used as positive controls in the study (Chapter 2). The zebra samples, positive for BPV-1, also clustered in this branch. The equine sarcoid described by Chambers *et al*. (2003) is descibed as BPV-1 subtype IV and only shows 97% similarity to the above sequences. However, other BPV-1 subtypes formed a separate branch. None of the giraffe or sable samples cluster in the BPV-2 branch, only BPV DNA obtained from zebras that were sequenced previously (Chapter 2). Although these animals occurred over a large geographical area, Mpumalanga, Northern Cape and Western Cape, the virus found in the different species is 99-100% similar. Cross-species infections therefore, do take place and they are not strictly species-specific as was thought earlier.

Clinical manifestation of latent infection in the three animals described here may have been provoked by stress. In the case of the sable antelope, this may have been that it was not in the type of habitat to which it was accustomed which induced immunosuppression. In the case of the giraffes one can only speculate that drought conditions and the resultant high tannin content of the *Acacia*-trees as a result of over browsing could act as a co-factor in BPV-associated carcinogenesis as seen in cattle following bracken fern (*Pteridium aquilinum*) ingestion (Campo 1997).

In conclusion, nodular fibropapilloma lesions in different species can be associated with bovine papillomavirus and cross-species infections do take place. The real-time PCR method could detect and distinguish between BPV-1 and -2 infections in these species. In free-living wild animals the course of the disease is difficult to monitor and interplay between external co-carcinogens and immunosuppressant compounds and the viral genes cause lesions to progress to cancer. When these animals are noticed the cancer is already widespread, as seen in the giraffes.

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