DETECTION AND CHARACTERIZATION OF PAPILLOMAVIRUS IN ZEBRA (*Equus zebra*) AND OTHER SOUTH AFRICAN WILDLIFE SPECIES

By

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Dedicated to my sons Wim and Gerhard

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SUMMARY

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For the **PhD degree**

Sarcoid-like tumours have been reported in Cape mountain zebra *(Equus zebra zebra)* in two South African game parks recently. These tumours caused severe distress to the animals and also made them unsightly for tourists visiting the parks.

The aim of this investigation was to identify and characterize the infectious agent considered to be involved in the aetiology of sarcoid in the Cape mountain zebra. Bovine papillomaviruses (BPV) -1 and -2 deoxyribonucleic acid (DNA) were detected by the polymerase chain reaction (PCR) in sarcoid tumour tissue, but not from blood specimens or unaffected skin. Differentiation between BPV-1 and -2 was made by using the restriction endonuclease *Bst*XI on PCR products of the E5 open reading frame (ORF).

A hybridization probe real-time assay was developed for the specific and sensitive detection and differentiation of BPV-1 and -2 DNA in blood, skin and sarcoid tumour samples. For the specific detection of BPV-1, an increase in fluorescence was detected at 640 nm and of BPV-2 at 705 nm. The test is extremely sensitive and able to detect 1.5 genome copies/reaction.

The presence of BPV-1 and -2 DNA could be demonstrated in the blood of sarcoid-affected and -unaffected zebras even in the blood of zebras from parks where sarcoids have never been observed.

The phylogenetic relationships of the papillomaviruses detected in tumours in the Cape mountain zebra in comparison with a broad selection of papillomavirus sequences available in GenBank were compiled. The papillomavirus sequences retrieved from the zebras were identified as variants of either BPV-1 or BPV-2. The age of the most recent common ancestor for BPV-1 variants is estimated to be 1.40 million years (Mya) and for BPV-2 variants, 0.55 Mya. The age of the most recent common ancestor of BPV-1 and BPV-2 is estimated to be 5.34 Mya.

Certain major histocompatibility (MHC) haplotypes are associated with increased risk of sarcoid tumours in horses. The zebras in these parks may have become inbred for the MHC region with increased prevalence for a haplotype, conferring increased risk for sarcoid tumours. Therefore typing system was developed to determine whether or not a high prevalence of sarcoids among zebras is associated with a MHC haplotype. Single strand conformational polymorphism was used to assess the genetic variation in MHC class II genes. The use of DQB and DRB genes demonstrated that genetic variation and sarcoids in the zebras could not be attributed to a specific haplotype.

The developed real-time PCR technique was also applied in the detection of cutaneous papillomavirus in two giraffes (*Giraffa camelopardalis*) which were manifesting cutaneous papillomatosis, in the Kruger National Park and in a fibropapilloma in a sable antelope (*Hippotragus niger*), on a game farm in the Kimberley district, South Africa.

In conclusion, this was the first study to confirm the presence of BPV-1 and -2 DNA in the sarcoid tumours, healthy skin and blood of sarcoid-affected and healthy free-roaming zebras from sarcoid-affected parks. The presence of BPV-1 and -2 DNA in the blood of zebras from parks where sarcoids have not been previously observed was a significant finding.

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CHAPTER ONE:

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

The Cape mountain zebra (*Equus zebra zebra,* Linnaeus 1758) is regarded as one of the most endangered large mammals in South Africa (Novellie *et al.* 2002) and the estimated extant 600 individuals (Glenn 2006) are protected in small numbers in reserves of which most are situated in their natural habitat. In two of the smallest of these reserves, Bontebok National Park (3 486 ha) and Gariep Dam Nature Reserve (6 000 ha) respectively, the presence and incidence of sarcoids were reported in 1995 and 1998 (Lange 2004; Nel *et al.* 2006). These were the first records of this most common tumour of horses and donkeys in free roaming zebras.

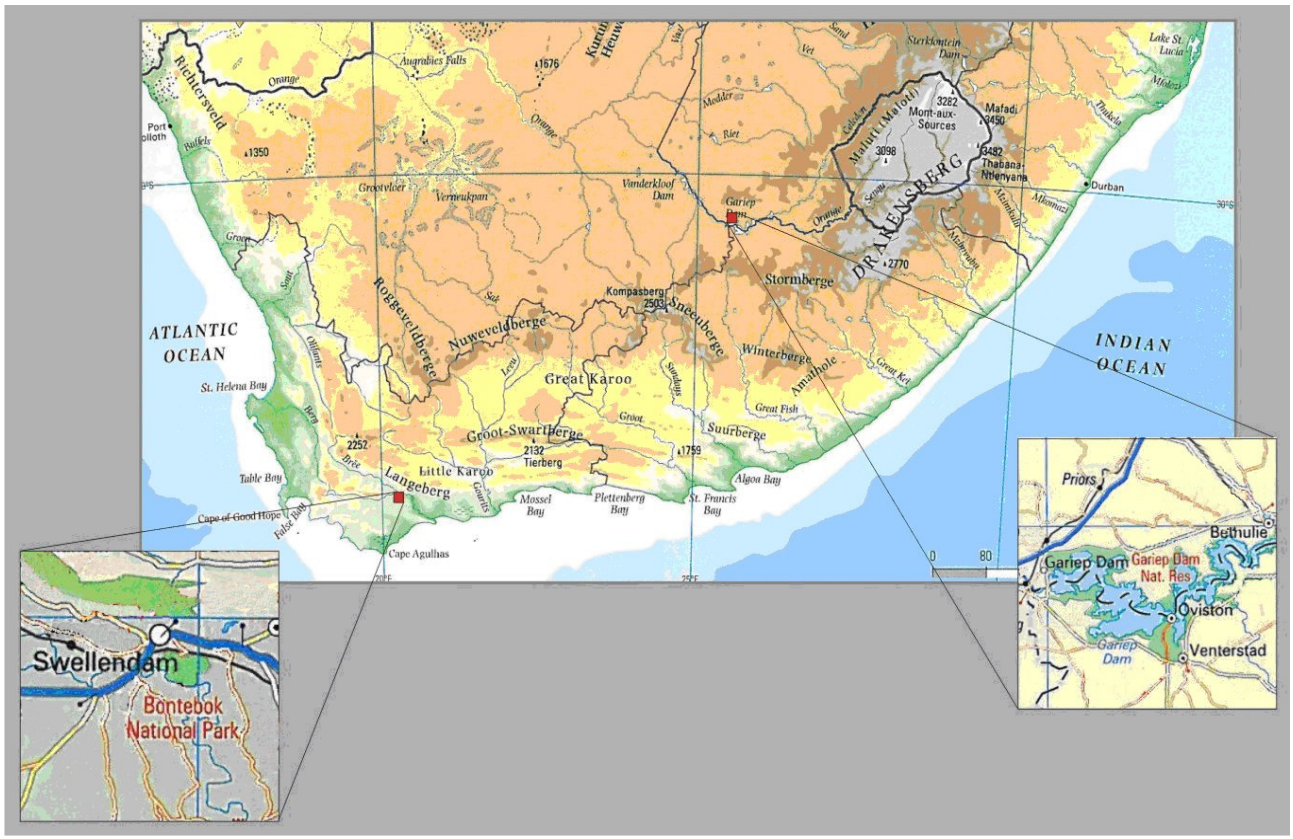

Figure 1 Geographical map of southern Africa showing the location of Gariep Dam Nature Reserve in the Free State and Bontebok National Park in the Western Cape Province. (Adapted from: MapStudio: Southern East Africa Road Atlas and Oxford Secondary Atlas for South Africa. Wiegrand P, (ed).

The Bontebok National Park is situated in the winter rainfall area, next to the Langeberg near Swellendam, Western Cape on the banks of the Breede River and Gariep Nature Reserve is situated in the arid southern Free State bordering the Gariep Dam (**Figure 1**). The suitability of the two parks for Cape mountain zebras is questionable since a habitat analysis of the Bontebok National Park in the Western Cape Province (**Figure 1**) showed that the general habitat is marginal for the maintenance of Cape mountain zebras (Kraaij & Watson 2009) and the Gariep Dam Nature Reserve, situated in the southern part of the Free State Province along the northern shore of the Gariep Dam, is sparsely covered with vegetation classified as eastern mixed Nama-Karoo (Low & Rebelo 1996). This is the most northern area in South Africa where Cape mountain zebras occur, their natural habitat being more to the south, and more closely confined to mountainous areas that offer the required types of grazing and shelter in the form of kloofs and ridges (Smithers 1983). In both of these parks the grazing is shared with other herbivore species.

The Bontebok National Park was proclaimed in 1931 to prevent the last few remaining bonteboks (*Damaliscus dorcas*) from becoming extinct. The rare red hartebeests (*Alcelaphus buselaphus*) also occur here. The ecological management plan of the Gariep Nature Reserve (proclaimed in 1985) recommends that the numbers of zebras are kept between 50 and 70 for this reserve (Nel *et al*. 2006). Other game species such as springboks (*Antidorcas marsupialis*), klipspringers (*Oreotragus oreotragus*), elands (*Taurotragus oryx*), kudus (*Tragelapus strepsiceros*), gemsboks (*Oryx gazella*), swart wildebeests (*Connochaetus gnou*), and rooi hartebeests (*Alcelaphus buselaphus*) also share the available grazing in the park.

Cape mountain zebra numbers dwindled to those in a few isolated populations, and all the existing Cape mountain zebras are descendants of a nucleus of 11 individuals (Bigalke 1952). They were and still are protected in the Mountain Zebra National Park in Cradock in the Eastern Cape Province, South Africa, from where they were reintroduced into yet other reserves. The zebras within these reserves also form closed herds and have become highly inbred.

Zebras are resistant to equine viral diseases such as African horsesickness virus, equine herpesvirus, equine encephalosis virus, equine arteritis virus, and although antibodies against several viral diseases have been demonstrated, clinical diseases were not seen (Barnard 1993; 1994; Blunden *et al.* 1998; Borchers *et al.* 2005). Zebras can be a source of African horsesickness virus, as the virus over winter in the zebra while they show no clinical signs of the disease (Erasmus 2008). Recently a high incidence of sarcoid lesions appeared in two parks, Bontebok National Park (**Figure 2**) and Gariep Dam Nature Reserve (**Figure 3**) affecting 53% and 24% of the Cape

mountain zebra populations respectively (Lange 2004; Nel *et al*. 2006). Characterization of the cause of these sarcoid forms the main emphasis of this study.

Figure 2 Sarcoid lesions in the axilla of a Cape mountain zebra in Bontebok National Park.

Figure 3 Sarcoid lesion seen on the ventral aspect of the jaw in a Cape mountain zebra in Gariep Dam Nature Reserve.

Bovine papillomaviruses types 1 and 2 (BPV-1 and BPV-2) are associated with sarcoids, the most common dermatological skin lesion in equidae (Goodrich *et al.*1998) and have been detected in donkeys (Reid *et al.* 1994), mules (Jackson 1936), horses (Otten *et al*. 1993), and two cases were reported from captive zebra in the United States of America (Löhr *et al.* 2005).

Papillomaviruses are oncogenic viruses which infect epithelial cells causing hyperproliferative lesions. They infect cutaneous or mucous epithelia in a variety of hosts. Bovine papillomaviruses 1 and 2, were found not to be as species-specific as other papillomaviruses which only infect their natural hosts.

Sarcoids in horses have been associated with major histocompatibility complex class II, with serological determinants to the DR and DQ loci, although the precise cause has never been determined (Lazary *et al.* 1988; Marti *et al*. 1996). The zebras in the reserves, being inbred, might have become inbred for the MHC region with increased prevalence of a haplotype conferring increased risk for sarcoids tumours.

Other free-roaming game species, with wart-like skin lesions, such as giraffes (*Giraffa camelopardalis*), have also been observed in the Kruger National Park, which is situated on the eastern border of South Africa bordering Mozambique, the wart-like skin lesions, often impairing their well-being and making them unsightly. A sable antelope (*Hippotragus niger*), kept on a game farm in the Kimberley district, Northern Cape Province, South Africa, developed a similar lesion on one of its legs. This arid area of South Africa is not the natural habitat of these antelopes as they are a savanna woodland species (Smithers 1983). Electron microscopically, virus particles have also been demonstrated in skin papillomas in an impala (*Aepyceros melampus*) and a giraffe in Kenya (Karstad & Kaminjolo 1978).

The aims of this study were:

- To investigate if bovine papillomavirus is associated with sarcoids in free-roaming Cape mountain zebras by using polymerase chain reaction and restriction fragment length polymorphism.
- To develop a real-time polymerase chain reaction diagnostic method to detect and distinguish between BPV-1 and -2.

- To study the phylogenetic relationships of the papillomaviruses detected from sarcoids in the Cape mountain zebra in South Africa in comparison with sequences of papillomaviruses causing sarcoids in other equidae species.
- To determine whether or not the high prevalence of sarcoids in Cape mountain zebras is associated with a major histocompatibility complex haplotype.
- To determine electron microscopically if bovine papillomavirus is present in the papillomas in the skin of the giraffe (*Giraffa camelopardalis*) and sable antelope (*Hippotragus niger*) and if so, to detect and distinguish between bovine papillomaviruses 1 and 2 using the developed real-time polymerase chain reaction method.

1.2 LITERATURE REVIEW

1.2.1 Southern African zebras

1.2.1.1 The genus *Equus*

The genus *Equus* can be divided into three subgenera: *Equus,* the true horses, *Asinus,* asses and hemiones (onagers) and *Hippotigris* (Groves & Ryder 2000). The latter contains the zebras, which occur naturally in Africa. The zebra group can be divided into three further subgroups; *Equus zebra, Equus quagga* and *Equus grevyi.* *Equus zebra,* the mountain zebra, is represented by two subspecies, the Cape mountain zebra (*Equus zebra zebra* Linnaeus, 1758*)* and Hartmann's mountain zebra (*Equus zebra hartmannae* Matschie, 1898).

Equus quagga, the plains zebra, is grouped into six subspecies, depending largely on their striping patterns (Groves & Bell 2003), which include *inter alia* the now extinct true quagga (*Equus quagga quagga* Boddaert, 1785) and Burchell's zebra (*Equus quagga burchelli* Gray, 1824).

Equus grevyi, Grevyi's zebra, has only one living species, the Grevyi's zebra (*Equus grevy,* Oustalet, 1882), which occurs in East Africa (Groves & Ryder 2000; Groves & Bell 2003) and like *Equus zebra zebra* and *Equus hartmannae,* is monotypic (Groves & Bell 2003).

1.2.1.2 Distribution

1.2.1.2.1 Cape mountain zebra (*Equus zebra zebra*)

Historically Cape mountain zebras occurred throughout the mountainous areas of the Western and Eastern Cape Provinces from Paarl Rock eastwards to the Amatola Mountains in the Cathcart area and northwards to the Nuweveld, Suurberg and Stormberg mountains (Millar 1970). After facing near extinction, the State proclaimed the farm, Babylon's Toren, in the Cradock District, a National Park in 1937 with a founder population of five stallions and one mare (Penzhorn 1975). More animals were, however, donated from neighbouring farms, and all South African Cape mountain zebras in the National Park are descendants of a nucleus of 11 individuals from which the number of mares is not yet known with certainty, but only four animals appear to be mares (Bigalke 1952). From here animals are removed annually to re-establish breeding herds elsewhere within their original range (Penzhorn & Novellie 1993).

1.2.1.2.2 Hartmann's mountain zebra (*Equus zebra hartmannae*)

Historically Hartmann's mountain zebras occurred in Namibia in the mountainous transition zone between the Namib Desert in the west and the plateau in the east (Smithers 1983). At present they are still found throughout their historical range, and widespread establishment of artificial water points has allowed them to occupy previously unsuitable habitat (Moodley 2002). Hartmann's mountain zebras in Namibia did not go through a period of population reduction as the Cape mountain zebra. Large populations are maintained in state-protected areas, conservancies in communal land, private farmland and other state-owned land (Novellie *et al*. 2002).

1.2.1.2.3 Burchell's zebra (*Equus quagga burchelli*)

It is the consensus of opinion that Burchell's zebras never occurred south of the Orange river (Smithers 1983). They are a savanna species, partial to open areas of woodland, open scrub and grassland, where water is available. Naturally occurring populations are presently only found in reserved areas in the northern and eastern parts of South Africa, as well as Botswana, Namibia, Tanzania, Kenya, Mozambique, Zimbabwe and Zambia (Smithers 1983). In recent years they have however been widely introduced into private properties and parks on a country-wide basis. Their numbers were never threatened and they are a species of great adaptive flexibility.

1.2.1.3 Phenotype

The two species of mountain zebras differ from Burchell's zebras in that the black stripes on their heads and bodies are narrower and consequently more numerous. Their dark and light stripes especially on the rump are more equal in width, whereas Burchell's zebras have yellow- or greyish shadow stripes between the black stripes on the hindquarter. Mountain zebra have white under parts with a central narrow black stripe running from the anterior extremity of the sternum ventrally on the belly to the navel. The black body stripes fade out on the lower parts of the flanks. In Burchell's zebras the black body stripes continue around the under parts of the belly. The two species of mountain zebras possess a "gridiron" pattern which is formed by the black markings on the rump, from the anterior aspects of the ilea to the base of the tail; this being absent in the Burchell's zebras. Mountain zebras have a distinct dewlap and are smaller in body size than Burchell's zebras, which also lack a dewlap (Smithers 1983).

The main difference between the two mountain zebra subspecies is their size. The Cape subspecies is smaller than the Hartmann's mountain zebra. The stallions in the Cape mountain zebra are smaller than the mares whereas no difference in size occurs in the Hartmann's zebra (Groves & Bell 2003). The hind legs of the Cape mountain zebra have broader black stripes than those on the forelegs, whereas in the Hartmann's mountain zebras the black stripes are narrower and approximately equal in width, especially on the rump. There is a distinct orange tinge to the skin over the nose above the black muzzle tip in the Cape mountain zebras which is clearly distinguished from the Hartmann's mountain zebras which has a black muzzle but lacks the orange coloured suffusion. The mane of the Hartmann's mountain zebras comes further forward between the ears than that of the Cape mountain zebra (Novellie *et al*. 2002).

1.2.1.4 Status

Cape mountain zebra populations are still a matter of concern as habitat loss, droughts; hunting, competition with domestic livestock and interbreeding of the two subspecies impair their successful survival. The International Union for the Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Animals (http://www.iucnredlist.org) at present lists the species, *Equus zebra,* in the vulnerable category, whereas previously (2008) it was considered as endangered. *Equus zebra zebra* is listed in Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix I (threatened with extinction), while *Equus zebra hartmannae* is listed in CITES Appendix II (not threatened with extinction, but may become unless closely controlled). Although their numbers have slowly increased in recent years,

the genetic diversity and continued existence of Cape mountain zebras are of great concern as the uneven distribution of a few relatively large populations still makes them vulnerable (Novellie *et al*. 2002).

The estimated population of the Cape mountain zebras is about 600 while that of the Hartmann's mountain zebras is believed to be around 7 000 (Glenn 2006). The Cape mountain zebras are maintained in several areas (parks, reserves, wilderness areas or private land) within their historic range. The Gariep Dam Provincial Nature Reserve is the most distant northern area in which they are protected and this area is out of their historical range. They are however reported to do very well there (Novellie *et al*. 2002). Although the Hartmann's mountain zebra never occurred in South Africa in historical times, they have been introduced and are protected in the Richtersveld National Park, which borders Namibia and elsewhere on private properties and game reserves in the Northern Cape Province such as the Augrabies National Park, where a population of Namibian stock have been introduced. In Namibia the Hartmann's mountain zebra population is large and occurs in a large area and across a variety of land tenure systems (see above). Only about a quarter of the estimated population occurs within formally proclaimed conservation areas, while the remainder is on commercial livestock and game farms (Novellie *et al*. 2002).

1.2.1.5 Conditions affecting zebras

In earlier literature it was assumed that wild animals were healthier than domestic animals but it was only after the conservation of wildlife became widespread that important research in the field of infectious and parasitic diseases commenced (de Vos 1973). Free-living animals live in a balanced host-parasite relationship as clinical-healthy animals but should conditions change, for example under stress of capture or confinement, this relationship may change and evidence of disease of the animal becomes apparent. The incidence of mortality from parasitism or infectious disease is increased by hyperactivity of the adrenal cortex (Christian 1963; Southwick 1969). Furthermore inbreeding is considered a factor which may lead to exacerbated susceptibility to pathogens (Frankham 2003).

Cape mountain zebras harbour large numbers of helminth parasites (Krecek *et al*. 1994), as well as ticks (Young *et al*. 1973; Penzhorn 1984; Penzhorn & Horak 1989).

Bacterial diseases affecting zebras, although rare, are anthrax (*Bacillus anthracis)* (de Vos 1994) and brucellosis (*Brucella abortus*) (Radcliffe & Osofsky 2002) and protozoal diseases such as trypanosomosis, babesiosis, toxoplasmosis and sarcocystosis (Radcliffe & Osofsky 2002).

The most important viral disease of zebras is African horsesickness (AHS) (Erasmus *et al.* 1978b), because it is the viral disease which causes the greatest mortality rate in horses and imposes a severe restriction on the movement and export of horses, affecting especially the horse industry. Of all equidae, zebras are the most resistant to the disease (Coetzer & Erasmus 1994), the duration of the viraemia can range from 11-30 days (Barnard 1993, 1994, Barnard *et al.* 1994) and, although not clinically ill, transport of such animals can lead to very serious consequences such as the introduction of AHS into Spain in 1987 when Hartmann's zebras were shipped from Namibia (Rodriguez *et al*. 1992). The overwintering of AHS horses from one year to the next in young zebra foals was demonstrated by Barnard (Erasmus 2008) which emphisises the important role of zebras in the disease.

Equine herpesvirus (EHV) infection appears to be widespread in free-ranging zebras and they may be a natural host of EHV-9, although there is no evidence of mortality due to this virus (Borchers & Frölich 1997; Blunden *et al*. 1998; Borchers *et al*. 2005; 2006.) and it is thought that equine herpesvirus infection is endemic in zebras in the Kruger National Park (KNP) (Barnard & Paweska 1993). The presence of antibodies to equine encephalosis virus (EEV) was demonstrated in zebras by Barnard and Paweska (1993). No evidence of infection with equine arteritis virus could be found in South Africa (Barnard & Paweska 1993) although antibodies were found in Burchell's zebras (*Equus quagga burchelli*) from the Serengeti ecosystem (Borchers *et al*. 2005) and some other southern African countries (Paweska *et al*. 1997). Reoviruses have also been isolated from zebras (Erasmus *et al*. 1978a) but no clinical disease reported.

Bovine papillomaviruses (BPV) are associated with equine sarcoids in horses (Angelos *et al*. 1991; Bloch *et al.* 1994; Chambers *et al.* 2003), and donkeys (Reid *et al*. 1994). Löhr *et al.* (2005) described such lesions in captive zebras (*Equus burchelli*) and such lesions have been reported in free-roaming Cape mountain zebras in game parks in South Africa (Lange 2004; Nel *et al.* 2006).

1.2.2 Sarcoid

1.2.2.1 Clinical appearance

Equine "warts" were first described in the 9th century AC by a stable master of the Caliph of Bagdad (Erk 1976), but the term "equine sarcoid" was first coined in South Africa in 1936 to describe a distinctive fibroblastic neoplasm occurring in the skin of horses, donkeys and mules (Jackson 1936) and to distinguish it clinically and histopathologically from papilloma, fibroma and fibrosarcoma.

The sarcoid is a unique, benign, non-metastasising but locally aggressive, fibroblastic (Jackson 1936) wart-like skin lesion that shows variable manifestations. It can occur on any part of the body, either singly or in clusters. The skin of the head, ventral abdomen and limbs are the most commonly affected sites (Goodrich *et al*. 1998). The paragenital region is also a common predilection site (Jackson 1936; Ragland *et al*. 1970). The gross appearance can vary and the tumour is, or can be classified into six distinct clinical types namely: occult, verrucous, nodular, fibroblastic, mixed (a mixture of the preceding four types) and malevolent (Knottenbelt 2005). Similarly the histopathological characteristics have been described for these clinically very different tumours (Ragland *et al*. 1966) and are requisites for their diagnosis (Goodrich *et al*. 1998). The typical appearance includes the dermal proliferation of fusiform or spindle shaped fibroblasts which form whorls of interlacing bundles. At the dermo-epidermal junction, a perpendicular orientation of fibroblasts towards the basement membrane of the covering epidermis (picket fence) is considered characteristic (Ragland *et al*. 1966).

Equine sarcoids bear no relation to human sarcoidosis (Stannard & Pulley 1978) and are the most common dermatological lesions of domestic equidae (Goodrich *et al*. 1998) apart from trauma-induced lesions. They affect horses (Angelos *et al*. 1991; Bloch *et al*. 1994; Chambers *et al*. 2003), donkeys (Reid *et al*. 1994; Nasir *et al*. 1997; Nasir & Reid 1999), mules (Jackson 1936), and zebras (Löhr *et al*. 2005; Nel *et al*. 2006). Reference to sarcoids in the Asian wild asses (hemiones / onagers) could not be found. Equines of all ages, types, breeds and colours are affected worldwide. Sullins *et al*. (1986) reported that sarcoid represent the most common neoplasm of all equine tumours, with reported prevalence rates ranging from 12.9-67%. However Knottenbelt (2005) has reorted a prevalence of sarcoid ranging from 1% to 8% worldwide.

1.2.2.2 Treatment

Currently no effective therapy for the treatment of sarcoids has been described. Various treatment methods are practised but the assessment of those in terms of success or failure is difficult as the course of the condition in many cases is not followed up as many are referral cases to major veterinary hospitals. The various forms of treatment described are: Surgical removal (Knottenbelt *et al*. 1995), but the major problem of this is regrowth of the tumour in the majority of animals at the same site (Ragland *et al*. 1970; Vingerhoets *et al*. 1988; Knottenbelt *et al*. 1995). Cryosurgery has been used in which some success has been obtained (Lane 1977; Laursen 1987; Goodrich *et al*. 1998). Laser surgery has also been used (Vingerhoets *et al*. 1988) and did not have the complications associated with cryosurgery such as swelling, hyperaemia, haemorrhage and local oedema (Vingerhoets *et al*. 1988). Immunotherapy as treatment comprises the intra-lesionally inoculation of five to nine weekly administrations of bacillus Calmette-Guerin (BCG) (Knottenbelt 2001), an attenuated strain of *Mycobacterium bovis*. The latter is a non-specific immunomodulator and is used in the therapy of sarcoids, good results have been obtained in the case of smaller tumours (Webster & Webster 1985; Vanselow *et al*. 1988), but the inflammatory reaction following its occulation can be a disadvantage. Chemotherapy in the form of topical treatment or implants has been used. It comprises the topical application or inoculation of one of or a mixture or a variety of heavy metals and the antimitotic compounds 5-fluorouracil and/or thiouracil (Turrel 1985). Intra-lesional inoculation of cisplastin in sesame oil has also been effective in 18 out of 19 sarcoid cases treated (Théon *et al*. 1993).

Marais (2006) used four different treatment methods: surgical excision, intralesional 5-fluorouracil treatment, subcutaneous administration of an autogenous vaccine prepared from tumerous tissue, and a combined intra-lesional 5-fluorouracil treatment with subcutaneous administration of an autogenous vaccine as treatment in the sarcoid-affected Cape mountain zebras in Bontebok National Park, Western Cape, South Africa. The vaccine comprised of 15 g of minced and filtered sarcoid tissue obtained from affected Cape mountain zebras, 84ml glycerol-saline (50% glycerol and 50% 0.9% NaCl), 1ml 10% formalin, 150 000 IU sodium penicillin and 100 mg dihydrostreptomycin. The treated zebras in the Park were inoculated once subcutaneously with 1 ml of the autogenous vaccine. The results were evaluated 18 months later. No evidence was found of sarcoid in the treated zebras except in one zebra which was treated with only 5-fluorouracil intra-lesional. This zebra developed several other sarcoids and was euthanized.

1.2.2.3 Viral aetiology

The first report suggesting a possible viral aetiology for sarcoids (Jackson, 1936) was based on their appearance and pattern of spread which, was proposed, was either by means of indirect contact with infested riding equipment or by direct contact with an affected animal. Development of sarcoids, in areas of prior skin injury, usually after several months, has also been reported (Voss 1969; Ragland *et al*. 1970). The viral hypophysis was further supported by the occurrence of apparent outbreaks among horses and mules in certain areas and within stables (Ragland *et al*. 1966; 1970). Transmission studies, in which either sarcoid tissue or cell-free supernatant from minced tumour, was inoculated intradermally into the skin of healthy horses, resulted in the appearance of tumours at the inoculation site which could not be differentiated from sarcoids (Voss 1969). Intradermal inoculation of BPV into horses (healthy as well as sarcoid-affected) led to the growth of tumours resembling sarcoids (Olson & Cook 1951; Ragland & Spencer 1969; Lancaster *et al*. 1977) which, in contrast with naturally-occurring sarcoids, spontaneously regressed in several horses. Regressions were complete within 12 days after the tumours were detected, but in others, they had only undergone either complete regression or were histologically in the late stages of regression, after a year. Bovine papillomavirus-neutralising antibodies could however be detected in the serum of the experimental horses (Broström *et al.* 1979; Ragland & Spencer 1969; Lancaster *et al*. 1977).

Lancaster *et al*. (1979) and Amtmann *et al*. (1980) demonstrated BPV deoxyribonucleic acid (DNA) in sarcoids using DNA hybridization techniques, but when polymerase chain reaction (PCR) detection methods were developed, they offered a far greater sensitivity. The presence of BPV DNA was demonstrated in sarcoids by several workers (Trendfield *et al*. 1985; Angelos *et al*. 1991; Lory *et al*. 1993; Otten *et al*. 1993; Reid *et al*. 1994; Bloch *et al*. 1994; Martens *et al*. 2001a; 2001b; Carr *et al.* 2001a; 2001b; Chambers *et al*. 2003; Bogaert *et al*. 2005; 2008; Nasir *et al*. 1997; Szczerba-Turek *et al*. 2010). Bovine papillomavirus DNA was also demonstrated in the normal skin of sarcoid affected animals by Carr *et al*. (2001a) who postulated that BPV might reside latently in fibroblasts until a factor such as trauma, initiates its transforming activity. It was previously thought that BPV DNA could not be demonstrated in sarcoid-free horses or in other skin lesions in horses (Angelos *et al*. 1991; Otten *et al*. 1993; Teifke 1994). It was, however, recently demonstrated by Bogaert *et al*. (2005; 2008) that BPV DNA occurred in swabs taken from the skin of unaffected horses. Bovine papillomavirus DNA has also been found in some cases of dermatitis (Angelos *et al*. 1991; Chambers *et al*. 2003; Yuan *et al*. 2007). It was shown in humans that human papillomavirus (HPV) type 5 plays a role in the pathology of psoriasis and that such lesions may be a reservoir for the virus (Favre *et al*. 1998; Majewski *et al*. 1999), which supports the hypothesis that inflammatory skin conditions may harbour papillomavirus (Nasir & Reid 2006). The role of

granulomatous inflammation in the pathogenesis of sarcoids is, however, not clear (Nasir & Reid 2006).

Despite the fact that BPV DNA was consistently demonstrated in equine sarcoid lesions, intact papillomavirus particles have never been demonstrated by electron microscopy, and the disease is considered to be a non-productive infection in which the viral DNA exists in an episomal form (Amtmann *et al*. 1980; Lancaster 1981). Bovine papillomavirus DNA has been demonstrated in whole blood of infected cattle (Campo *et al*. 1994a; Campo 1998; de Freitas *et al*. 2003; Wosiacki *et al*. 2005). Blood transfusions from papilloma-affected cattle to healthy cows resulted in the detection of BPV DNA in their peripheral blood mononuclear cells (PBMCs) of such cows as well as those of their progeny, thus supporting the concept that BPV can be transmitted *in utero* (Stocco dos Santos *et al*. 1998). Until recently, BPV DNA had not been demonstrated in the blood of BPV-infected horses and donkeys (Nasir *et al*. 1997). Brandt *et al*. (2008), however, demonstrated the presence of BPV DNA in PBMCs obtained from sarcoid-affected horses but not in those of the control horses which were derived from healthy animals.

1.2.2.4 Disease transmission

The mode of BPV transmission within and between equines is still unclear. In cattle, BPV is transmitted by contact between animals or contact with fomites (Campo & Bastianello 2004). Cutaneous papillomas appear particularly in sites such as the forehead and neck in groups of calves, which are the sites that are particularly subjected to trauma when the animals are confined in groups and rub against hayracks and posts, which become contaminated with BPV, leading to infection of healthy animals subsequently introduced into the pens (Jarrett 1985). Vertical transmission in cattle was postulated by Stocco dos Santos *et al*. (1998) following the detection of BPV-2 DNA and chromosomal alterations in the peripheral blood of offspring at the moment of birth. Currently, it is not known whether BPV infection of horses is transmitted from one horse to another, whether the virus is transmitted from cattle to horses, or how BPV can cause infection in a non-natural host (Nasir & Campo 2008). The occurrence of epidemics of equine sarcoids in herds of horses and donkeys has also been reported (Ragland *et al*. 1966; Reid *et al*. 1994).

Nasir and Campo (2008) found evidence that indicated animal-to-animal transmission after housing pairs of healthy and sarcoid-infected donkeys together, although the authors did not specify for how long. They subsequently found that the healthy donkeys developed sarcoid and that the BPV sequences within each pair were identical but different between the pairs.

It is not clear if contact with cattle is a risk factor for the development of equine sarcoids (Jackson 1936; Mohammed *et al*. 1992). Bogaert *et al*. (2005), sampled horses in contact with cattle and concluded that this close association did not lead to an increased occurrence of BPV DNA in the horses.

Kemp-Symonds (2000) demonstrated BPV DNA in face flies (*Musca autumnalis*) collected from sarcoid affected animals but not from flies collected from healthy animals. This may imply that flies may act as vectors for the disease. Recently, Finlay *et al*. (2009) also demonstrated the virus in several different species of flies including biting and non-biting species trapped in the vicinity of sarcoid–affected horses. Sequence analysis confirmed BPV-1 in the flies and also supported the proposition that flies may act as vectors of disease transmission which was suggested by Torrontegui & Reid (1994).

1.2.2.5 Virus latency

The reactivation of BPV at sites of trauma also suggests that viral DNA is present in a latent form and that the skin damage induced expression of viral genes and consequent development of warts (Campo 1999). Sites of latency include normal epithelium and circulating lymphocytes (Stocco dos Santos *et al*. 1998) and in humans, human papilloma virus DNA has also been found in blood cells of women suffering from urogenital papillomas and cervical cancer (Pao *et al*. 1991; Kedzia *et al*. 1992). It was documented (Bodaghi *et al*. 2005) that human papillomavirus might be disseminated through the blood, the virus then serving as a source of infection for epithelial cells. In the horse, BPV DNA has been found in the normal skin of animals showing no sign of sarcoid (Carr *et al*. 2001a; Bogaert *et al*. 2005;) and in cattle BPV DNA has been demonstrated in the healthy skin indicating that the virus is present in a latent state in animals showing no clinical evidence of papilllomatosis (Borzacchiello 2003).

1.2.2.6 Immune suppression

The immune system plays an important role in the outcome of papillomavirus infections. Immune suppression and physical trauma can reactivate latent asymptomatic papillomavirus infections (Campo 1999). In humans immune suppression increases the risk of non-melanoma skin cancer (NMSC) considerably (Penn 1997; de Villiers 1998). Fair skinned patients who received immunosuppressive therapy after renal transplantation (Leigh & Glover 1995), or were infected with the human immunodeficiency virus (HIV) (Wang *et al*. 1995; Maurer *et al*. 1997) developed

NMSC much more readily. A systematic investigation of the immune response in horses and donkeys suffering from sarcoids has not been carried out.

Naturally-occuring sarcoids do not regress in contrast to other lesions caused by papillomavirus infections; this suggests that BPV infection in horses causes immune evasive mechanisms. The fact that experimentally-induced sarcoids can be caused to regress (Ragland & Spencer 1969) supports the supposition that naturally-occurring sarcoids may occur as a result of immune response. E5, the major oncoprotein of BPV, causes retention of the MHC class I molecule in the Golgi apparatus of infected cells (Ashrafi *et al*. 2002; Marchetti *et al*. 2002). This benefits the virus as the absence of the MHC Class I molecule from the cell surface causes the infected cells to evade host immunsurveillance as the MHC class I molecule is required for the presentation of viral antigens to CD8+ T-lymphocytes for the elimination of infected cells (Ashrafi *et al*. 2002; Marchetti *et al*. 2002).

1.2.2.7 Co-factors for carcinogenesis

If BPV overcomes the immune response of the host, as in immunocompromised animals, the lesions it causes persist and spread, thus placing the host in a high risk for the development of cancer. Infection of animals with certain papillomaviruses demonstrates the relationship between virus and environmental co-carcinogens. Cottontail rabbit papillomavirus (CRPV) and canine oral papillomavirus (COPV) and their interaction with chemical carcinogens, such as dimethylbenzanthracene, methylcholanthrene and tar have been recognised (Campo *et al*. 1994b; Breitburd *et al*. 1997). In humans ultraviolet light plays a role in the induction of epidermodysplasia verruciformis, a genetic disease, and certain factors in cigarette smoke play a role in the development of cervical cancer. Epidermodysplasia verruciformis is a rare genetic disease that results in increased susceptibility to HPVs (Orth 2006) and a high risk of carcinomatous transformation. Co-factors identified in BPV-associated carcinogenesis in cattle occur in bracken fern (*Pteridium aquilinum*). The virus (BPV) infects the bladder mucosa, and producing an abortive latent infection which is then activated by bracken-induced immunosuppression, which initiates the development to malignancy (Campo 1997). It has also been shown in humans that ingestion of the bracken carcinogen, ptaquiloside, in milk from bracken-fed cows, has been epidemiologically linked to gastric cancer (Alonso-Amelot & Avendano 2001). No co-factor has as yet been identified for BPV-associated equine sarcoids.

1.2.2.8 Genetic factors

1.2.2.8.1 Major histocompatibility complex

The major histocompatibility complex (MHC) glycoproteins play an important role in immune cell interactions. Co-dominant expression of class I and II allelic products and the high degree of polymorphism of these products in a species are thought to assure a survival advantage to the species by enhancing the ability of the immune system to interact with a great variety of pathogens.

Equine MHC glycoprotein was localized in the equine chromosome 20q14-q22 by *in situ* hybridization (Ansari *et al*. 1988; Mäkinen *et al*. 1989). Molecular techniques have made it possible to demonstrate the complexity of the system and to type the genes involved without the further use of serological techniques.

The MHC class I and class II genes play a key role in the initiation of the immune response (Klein 1986) by binding and presenting peptides to T-cells of the immune system. The MHC class I molecules present endogenously-derived peptides to cytotoxic T cells while MHC class II molecules present exogenously-derived peptides to T helper cells. The MHC class I and II molecules are both expressed on the surface of the antigen-presenting cells as heterodimeric molecules. Class I molecules are composed of a microglobulin (β_2m) and a heavy chain (α). The class II molecule is composed of a α and a β chain (Bailey *et al*. 2000). They are all encoded in the MHC region except β_2m, which is encoded by a gene on a separate chromosome (Andersson 1996).

Some MHC genes and gene products exhibit a high level of genetic polymorphism and are therefore one of the most well studied gene regions in vertebrate species (Anderson 1996). It is thought that the MHC diversity has evolved as a response to the selection pressure on the vertebrate immune system to recognize and eliminate invading parasites and micro-organisms. Genetic diversity of MHC molecules is a consequence of two mechanisms: gene duplication and allelic polymorphism. Peptide-presenting class II molecules are encoded by multiple loci. Even homozygotes possess multiple forms of antigen-presenting molecules as the presence of two or more linked loci, which encode distinct molecules, are indicated as “fixed heterozygosity” (Andersson 1996).

In certain animal populations a reduction in MHC diversity due to genetic bottlenecks and subsequent inbreeding may contribute to increased sensitivity to infectious pathogens (O’Brien & Yuhki 1999). Cases of papilloma infection in inbred populations were described by Van Ranst *et al*. (1991) who sequenced a novel papillomavirus, pygmy chimpanzee papilomavirus, in an

inbred pygmy chimpanzee (*Pan paniscus)* colony following an epidemic of a focal epithelial hyperplasia-like disease. A similar rare disease has also occurred in human populations such as the isolated Navajo Indian and Greenland Inuit populations. Human papillomavirus-13 was isolated, which showed an 83% sequence homology with the pygmy chimpanzee virus (van Ranst *et al*. 1992).

The MHC loci undergo selection. They are among the most polymorphic loci known in vertebrates; at a typical MHC locus there are numerous alleles of intermediate frequency in keeping theoretical predictions for balancing selection (Hedrick & Thomson 1983; Klein 1986).

The MHC molecules encoded by different alleles differ in their peptide binding capacities (Doherty & Zinkernagel 1975). An individual heterozygous at all, or most, MHC loci will be protected against a wider variety of pathogens than a homozygous individual. Reduced diversity of the MHC has been associated with disease in captive populations of wild animals, as demonstrated in captive populations of cheetah (*Acinonyx jubatus*) (O'Brien *et al*. 1985) and cottontop tamarins (*Saguinus oedipus*) (Watkins *et al*. 1988).

Further proof of the correlation between the MHC haplotype of an individual and the susceptibility of that individual to disease was present in chickens in which resistance to fatal infection of Marek's disease is associated with the B-21 haplotype (Briles *et al*. 1977) and in cattle the bovine leukaemia virus with the A14 bovine lymphocytic antigen (Palmer *et al*. 1987). In humans, certain human leukocyte antigens (HLA) class I and II alleles are strongly associated with susceptibility to over 40 human diseases, most of which are autoimmune in nature (Gregerson 1989; Bell *et al*. 1989). Amiel (1967), reported the association of Hodgkin's disease with HLA antigens. Other diseases include insulin-dependent diabetes mellitus and DR3 or 4 (Todd 1990), as well as ankylosing spondylitis and B27 (Brewerton *et al*. 1973).

1.2.2.8.2 Genetic predisposition to sarcoids

A hereditary predisposition of sarcoid affected horses to an exogenous virus was originally suggested by familiar patterns in the disease by James in 1968 and Ragland *et al*. in 1970. It was subsequently reported that Appaloosa, Arabian and Quarter Horses had a significantly higher risk of developing the tumour as opposed to Thoroughbreds (Angelos *et al*. 1988; Mohammed *et al*. 1992). Standardbreds, in contrast, were less than half as likely to develop sarcoids, or they were not observed at all in them (Meredith *et al*. 1986). As the disease revealed this tendency to develop in certain breeds and families (Broström *et al*. 1988), a genetic predisposition was suggested (Gerber

1989), which was based on the strong association between the prevalence of the tumour and genes in, or near, the equine MHC.

The first studies concerning a possible predisposition to sarcoids were conducted on the genetic variation of antigens attached to lymphocytes and, on the basis of family studies demonstrating linkage of a system of lymphocyte alloantigens to the A blood group system, the equine lymphocyte antigen (ELA) locus was defined (Bailey *et al*. 1979). Workshop meetings, to standardize the nomenclature and collaborate research, developed 21 serological specificities. Most belong to a single Class I MHC locus while the other appeared to be Class II. Developing serological tests for class II gene products was a major priority at the ELA workshops (Bernocco *et al*. 1987; Lazary *et al*. 1988). Lazary and co-workers (1980) demonstrated that the ELA system was the MHC of the horse which included both class I and II genes and that they were tightly linked. The series of class I antigens first described were denoted as locus A products. Later other antigens, encoded on other loci were assigned as locus B and C products. Antigens recognized in the workshop but of which the relationship to other alleles is not clarified have a prefix of W (workshop). Locally defined ELA antigens are designated with the abbreviation of the laboratory (Marti *et al*. 1996). Molecular studies demonstrated that variation of MHC class I genes is greater than that detected serologically (Bailey *et al*. 2000). The allelic class II antigens are denoted as products of the D region. It was demonstrated with DNA based molecular techniques that genes similar to the human homologs of DQA, DQB, DRA and DRB are present in the horse, and these loci are called the same as in man (Marti *et al*. 1996). The relationship of the serological determinants to the molecular DR and DQ loci remains unknown and therefore the “W” and “Be” still remain in the reference to the loci (Bailey *et al*. 2000).

The association of sarcoid tumours with the equine MHC was first reported by Lazary *et al*. (1985). Subsequently the association between ELA class I or II antigens and susceptibility to sarcoids has been established in different breeds in several countries. The frequency of the MHC classs II antigen DW13 was greater in sarcoid affected Swedish halfbreds, French, Irish and Swiss Warmbloods (Broström *et al*. 1988) and Thoroughbreds (Meredith *et al*. 1986), than that of the healthy controls. Broström (1995) also demonstrated an association between the early onset of sarcoids and the class I A5 haplotype as well as an increased incidence of recurrence of sarcoids after surgical invention in horses with the class II DW13 haplotype.

ELA-DW13 shows linkage disequilibrium with the class I alleles A3, A5 and A15. Therefore, these class I alleles may also play a role in the weak association with susceptibility to sarcoid (Bailey *et al*. 2000). In certain breeds, for instance the Standardbred, the allele DW13 does not occur, and sarcoids are very rare (Meredith *et al*. 1986). Despite the strong association of DW13 with sarcoid susceptibility among Warmblood horses, some possessing the allele do not develop the condition, while others that do not have the allele do developed it. Gerber and co-workers (1988) found that nearly all the affected offspring in one study inherited a particular paternal haplotype which is not found to be associated with the disease. Sarcoid susceptibility is associated with different class I or class II antigens depending on the breed or family studied, and such genes might act as genetic markers and a putative "sarcoid-susceptibility" gene might be located between these loci (Marti *et al*. 1996).

1.2.2.8.3 Genetic predisposition in other species

Papillomavirus-induced tumours possessing similar associations with MHC class II genes have also been encountered in other species. Cottontail rabbit papillomavirus- induced infections are more common in rabbits with a unique MHC class II haplotype (Han *et al*. 1992; 1994). Wank and Thomssen (1991) and Wank *et al*. (1993) showed that women carrying the HLA-DQW3 class II MHC allele have an increased risk of developing papilloma virus–induced squamous cell carcinoma of the cervix while in another study, an association was found between such tumours and HLA DR-DQ haplotypes (Apple *et al*. 1994). Apart from cervical cancer in humans certain HLA polymorphisms in the class II alleles may predispose carriers to specific papillomavirus-induced diseases such as recurrent respiratory papillomatosis (Breitburd *et al*. 1996; Gelder *et al*. 2003).

1.2.3 Papillomaviruses

Papillomaviruses are oncogenic DNA viruses which infect cutaneous and mucous epithelia in humans and a variety of animals. They are, however, strictly species-specific and, with one exception, only infect their natural host. The only cross-species infection known is that of equids by BPV types 1 and 2, the resulting lesion is known as a sarcoid (Nasir & Reid 2006). In the infected host papillomavirus infections induce papillomas which generally regress but occasionally persist and progress to malignancy.

1.2.3.1 Phylogeny of papillomavirus

Genomic organizations are similar in papillomaviruses of different animal species, and any pair contains at least five homologous genes, although the nucleotide sequence may diverge by more than 50% (Bernard 2006). Phylogenetic trees can be constructed on the basis of sequenced genes using computational phylogenetic methods.

Papillomaviruses and their vertebrate hosts have probably co-evolved over a period beginning 100 million years ago in which no change of host-species or any recombination has place; their basic genomic organization was maintained over this period of time. A phylogenetic tree of papillomaviruses is compatible with that of the infected mammalian species involved, including features such as clustering of the papillomavirus from distantly related ungulates, and the close association of papillomavirus from monkeys and apes with specific human papillomaviruses (Chan *et al*. 1992; van Ranst 1992). The origin of papillomavirus species specificity is not fully understood, but it may derive from the mechanistic specificity of molecular interactions (Shadan & Villareal 1993) or from the mode of transmission (Bernard 1994).

No molecular changes in papillomavirus genomes have been encountered seen since papillomavirus genome sequences were first published (±20 years ago) but it is estimated that papillomavirus genomes change at the same slow rates as their vertebrate host genomes: 1% nucleotide exchange per 100 000 to 1 000 000 years (Bernard 2006). Knowledge about viral evolution is still relatively poor compared to that of other organisms, and an extensive connection between evolution and phylogenetic relationships of papillomaviruses has not yet been developed (Gottschling *et al.* 2007).

1.2.3.2 Taxonomy of papillomavirus

The International Committee on Taxonomy of Viruses officially recognized taxonomy of papillomaviruses based on sequence comparisons from which the phylogenetic trees were constructed. All papillomaviruses belong to the family *Papillomaviridae.* Major branches of the phylogenetic tree are “genera”, indicated by Greek letters, and minor branches are “species”. The previous identification of types, subtypes and variants are not affected as they are currently grouped as “species” (de Villiers *et al*. 2004; Bernard, 2006).

The first papillomavirus types were isolated in the 1970s (Orth *et al.*1977; Coggin & zur Hausen 1979), but an appropriate cell culture in which to propagate these viruses to study their functions was not available at the time and taxonomy, based on their biological properties, could not be done (de Villiers *et al.* 2004). Raft cultures and xenograph modules (Kreider *et al.* 1985; Hummel *et al.* 1992;) were developed at a much later period but they had no major influence on the functional and taxonomic comparison of the papillomavirus types (de Villiers *et al*. 2004).

Papillomaviruses are classified according to the L1 genes (de Villiers *et al*. 2004), the classification being based on the traditional criterion that the sequence of their L1 genes should be at least 2-10%, dissimilar from one another.

1.2.3.3 The virion

Electron microscopically, spherical intranuclear papillomavirus particles of 55-60 nm with distinct capsomeres in the cells of the stratum granulosum of the skin of the infected region can be seen. Each consists of a non-enveloped icosahedral structure which forms paracrystalline arrays in the nucleus of infected cells. The nuclei display irregular clumps of condensed chromatin (Doane *et al*. 1987).

The virion contains double-stranded covalently closed circular DNA conjoined with cellular histones. The virion is comprised of major L1 and minor L2 capsid proteins. An atomic model of a BPV virion has been generated which demonstrates the exposure of the C-terminus of L1 and the N-terminus of L2 on the surface of the virion; it being likely therefore that they play a role in infection and immunogenicity (Nasir & Campo 2008).

1.2.3.4 The viral genome

All papillomaviruses have a similar genomic organisation. This is a double stranded circular DNA molecule of approximately 8 kilobases. The genome is divided into three canonical regions: a long control region, (containing the elements necessary for the replication and transcription of viral DNA), a region containing the early genes (encoding non-structural proteins) and a region containing the late genes (encoding the structural proteins) (Kalantari & Bernard 2006).

The transcriptional control of BPV depends on the interactions between the 12 sites in the long control region and the E2 protein, which is vital for the life cycle of BPV. Additionally, there are a number of cellular transcription factors which, in a fine balance, interact with the long control region and regulate the expression of viral genes. When this balance is destabilized, transcription of the viral genes, and expression of the viral proteins increase which result in a neoplastic state (Nasir & Campo 2008).

1.2.3.5 Oncoproteins

Bovine papillomavirus encodes three oncoproteins, E5, E6, and E7 of which the major viral oncoprotein is E5. It is a short hydrophobic membrane protein localizing on the Golgi apparatus and other intracellular membranes. It binds to, and activates, the platelet-derived growth-factor-β receptor (PDGF-R) in transformed cells, and this stimulation activates a receptor signaling cascade which results in an intracellular growth stimulatory signal (DiMaio & Mattoon 2001). It also causes retention of the MHC class I in the Golgi apparatus of infected cells (Ashrafi *et al*. 2002; Marchetti *et al*. 2002) which benefits the virus as the absence of the MHC Class I from the cell surface causes the infected cells to evade host immuno surveillance.

The E5 protein plays an important role in the pathogenesis of virally-induced papillomatosis in cattle and most of the studies concerning gene expression have focused on expression of this major BPV transforming gene. Deoxyribosenucleic acid sequences have been studied in donkeys (Reid *et al.* 1994) and horses (Chambers *et al.* 2003).

1.2.3.6 Sequence variation

Studies by Trendfield *et al*. (1985) and Angelos *et al*. (1991) were the first to report sequence variation in the bovine papilloma virus in sarcoids, and reported that the virus is closely related to BPV. More evidence of this followed (Otten *et al*. 1993; Reid *et al*. 1994; Chambers *et al*. 2003) and it is now established that sarcoids are associated with distinct variants of BPV or that an equine adapted variant of BPV exists that specifically infects equids.

Bovine papillomavirus infection of horses results in a different disease pathology when compared with that of cattle; the reason for this is unknown. Sequence variation in one or more of the proteins, E2, E5, E6, or E7, may lead to an altered biological function which affects the clinical outcome of the disease (Giannoudis & Herrington 2001).

1.2.3.7 Viral replication

In all papillomavirus infections that have so far been investigated, viral replication only take place in keratinocytes undergoing terminal differentiation to squamous epithelial cells. It is therefore only evident in the epithelial component of tumours and only in those in certain stages of the development. Viral replication has never been found in fibroblasts in which the BPV genome is present in a non-integrated episomal form (Jarret 1985).

Initial infection requires access of infectious particles to cells in primitive keratinocytes in the basal layer of the epidermis and squamous epithelia, the virus probably targeting stem cells (Stanley 2006). The virus maintains its genome as a low copy number episome in the basal cells of the epithelium (Doorbar, 2005). In uninfected epithelium, basal cells exit the cell cycle soon after migrating into the suprabasal cell layers and undergo a process of terminal differentiation whereas, when infected, this normal terminal differentiation is retarded. The virus must escape from the infected cell and survive extra-cellularly before reinfection. Papillomaviruses are non-lytic and are only released when infected epithelial cells reach the epithelial surface. This retention of the virus may compromise its immune detection as well as the molecular mechanisms that limit the presentation of viral epitopes to the immune system in the lower epithelial layers (Ashrafi *et al*. 2002). Canine oral papillomavirus differs in this aspect as genome amplification begins as soon as the cells leave the basal layer (Doorbar 2005).

In the basal layer of keratinocytes, the E5 protein is expressed intracytoplasmically at very low levels but in differentiated keratinocytes large amounts of oncoprotein are expressed (Burnett *et al*. 1992).

1.2.3.8 Malignancy

The fact that human infection with certain specific papillomaviruses is the most important risk factor in the development of cervical cancer, leads to the concept that papillomaviruses indeed are aggressive neoplastic agents. Considering the high incidence of papillomaviruses presently associated with normal skin (Antonsson *et al*. 2003) but rarely associated with neoplastic conditions (de Villiers 1998), the occurrence of neoplastic disease is an extremely rare event.

The development of malignant neoplasms is the end-point used to assess carcinogenicity in the study of human papillomavirus infections, but in mammalian species of animals the induction of benign tumours (warts and papillomas) is often the end point used for this purpose in both natural and experimental infections. The incidence of warts is higher than that of cancer and is easier to monitor because benign neoplasms are generally less life threatening than their malignant counterparts. It is difficult to monitor the course of a disease in free-living wild animals. Domestic farm animals are usually slaughtered before the onset of malignancy. Papilloma-associated cancer ultimately derives from warts, the presence of which can therefore be an indication of possible initial neoplastic development (IARC Monographs).

In advanced cases, transformed cells are possibly no longer permissive to virion production, and one of the several outcomes may be expected: the viral genome is incorporated into the cellular genome as in human ano-genital squamous cell carcinoma, maintained as an extrachromosomal element which replicates in synchrony with the cell cycle, as in urothelial cancers in cattle, or may be lost by the transformed cells, as in squamous cell carcinomas of the upper gastrointestinal tract in cattle (Campo 2006a).

When the papilloma has undergone malignant transformation to carcinoma, the structural integrity of the virus is lost and virus or viral structural antigen is not present. Similarly, tumours induced in unnatural hosts as well as cell lines established from these tumours contain no detectable mature virus (Lancaster & Olson 1982).

The progression of some viral-induced lesions to malignancy is undesirable even for the virus itself, because chromosomal integration of the papilloma genome is irreversible and death of the host terminates viral replication (Bernard 1994).

In the work by Campo *et al.* (1994b), it was found that the continuous presence and expression of the viral genome is not necessary for the maintenance of the neoplastic state. Interplay between external co-carcinogens and immunosuppressant compounds and the viral genes is necessary for the development of virus lesions induced by the papillomavirus and their progression to cancer (Onions 1997).

1.2.3.9 Papillomavirus infection in other species

Histopathologically tumours, similar to equine sarcoid, were diagnosed by Teifke *et al*. (2003) in 12 feline skin lesions. They differed in their histological appearance, site of development and behaviour from those of feline papillomas, which are caused by host-specific feline papillomaviruses (Sundberg *et al*. 2000). The presence of papillomavirus itself was not demonstrated by immunohistochemistry in these lesions, but papillomavirus DNA was detected in nine of the 12 samples investigated. Schulman *et al*. (2001) have reported the detection of papillomavirus in feline tumours that had clinicopathologic features similar to those of equine sarcoids.

Close relationship in sequence similarity between species has been shown by Munday *et al*. (2009), who amplified papillomavirus DNA isolated from oral squamous cell carcinoma of a cat and revealed that it was most similar (92% similarity) to human papillomavirus type 76, which only causes disease in immunosuppressed humans (Orth 2006). Tachezy *et al*. (2002) isolated papillomavirus type 1 from cutaneous viral plaques in a domestic Persian cat, which had a close relationship in sequence similarity to canine oral papillomavirus and Munday *et al*. (2007a) isolated DNA that was most similar (98%) to HPV type 9 in a similar lesion on a domestic Shorthair cat. Is unknown whether HPV type 9 was passed from human to cat or whether HPV type 9 lives asymptomatically in cats and occasionally causes disease in people. In another study Munday *et al*. (2007b) detected multiple papillomavirus types in feline Bowenoid *in situ* carcinomas.

Antonsson *et al*. (2000) showed that 80% of people are asymptomatically infected with betapapillomaviruses, a major branch of the phylogenetic tree of papillomaviruses. It was also shown that skin papillomaviruses could be detected in healthy skin from many different animal species, and that they were sufficiently genetically related to their human counterparts to be identifiable by a human skin papillomavirus primer set (Antonsson & Hansson 2002). Furthermore Antonsson & McMillan (2006) showed that the range of species infected by papillomaviruses extends to most primitive mammals: the monotremes and the marsupials.

Papilloma infections in the oral cavity of six feline species, (Asian lion, *Panthera leo,* P1PV; snow leopard, *Panthera unica*, PuPV-1; bobcat, *Felis rufus*, FrPV; Florida panther, *Felis concolor,* FcPV; clouded leopard, *Neofelis nebulosa*, NnPV; and domestic cat, *Felis domesticus,* FdPV-2) and the skin of two species: (domestic cat, *F domesticus*, FdPV-1; and snow leopard, *P unica* PuPV-2) have been reported by Sundberg *et al*. (2000). Schulman *et al*. (2003) and Sundberg *et al*. (2000) identified eight new papillomavirus types in these six different feline species, which were held

either in captivity or were free ranging. Of the animal papillomaviruses, only COPV has been studied extensively but other uncharacterised papillomaviruses have also been isolated from dogs, their relationship being closest to human and bovine papillomaviruses (Zaugg *et al*. 2005). The latter study also determined that COPV DNA is rarely present in squamous cell carcinoma tissues as it was only demonstrated in 2.3% of the samples studied. Similarly COPV DNA was only amplified in 6% of squamous cell carcinoma in tissue samples examined in another study (Teifke *et al*. 1998) in which it was demonstrated that a higher percentage of papillomavirus antigens (27%) can be determined in immunohistochemistry studies (Schwegler *et al*. 1997). The presence of several different papillomavirus types in the canine squamous cell carcinoma exist, and dogs like humans, can be infected with papillomaviruses of great genetic diversity.

Electron microscopically, virus particles have also been demonstrated in skin papillomas in an impala (*Aepyceros melampus*) and a giraffe (*Giraffa camelopardalis*) in Kenya (Karstad & Kaminjolo 1978).

Camelid mucocutaneous fibropapillomas, from alpacas (*Vicugna pacos*) and llamas (*Llama glama*), have been shown to contain an identical papillomavirus whose 176bp E1 gene fragment has been cloned and sequenced. It was found that it is a unique papillomavirus with 73% homology to BPV-1 and a 64% homology to canine papillomavirus (Schulman *et al.* 2003).

Bovine papilloma virus type 2 was described by Literák *et al*. (2006) in a European bison (*Bison bonasus*) and Silvestre *et al*. (2009) isolated BPV-1 from the water buffalo (*Bubalus bubalus*).

Bovine papillomavirus is the most extensively studied animal papillomavirus. Bovine papillomaviruses are responsible for exophytic papillomas of both cutaneous and mucosal epithelium in cattle. It is an agent of disease in farm animals and of veterinary importance, although they have been studied as a relevant model of human papillomavirus (Borzacchiello & Roperto 2008). They are benign tumours and generally regress without serious problems to the host but they can persist leading to squamous cell carcinoma especially in the presence of environmental co-factors (Campo 2006b).

Initially six different types of BPVs were distinguished on the basis of DNA sequence relatedness. Each BPV is associated with type-specific lesions: BPV-1 and BPV-2 infect the epithelium and dermis, giving rise to fibropapillomas; BPV-3, BPV-4 and BPV-6, are strictly epitheliotropic, inducing true epithelial papillomas, and BPV-5 infects the epithelium and the dermis, inducing both fibropapillomas and true epithelial papillomas of the skin (Campo 2002; de Villiers *et al*. 2004; Hatama *et al.* 2008). Subsequently BPV-7 (Ogawa *et al.* 2007), BPV-8 (Tomita *et al.* 2007), BPV-9 and BPV-10 (Hatama *et al*. 2008) were sequenced and phylogenetically analysed.

The highly endangered Florida manatee (*Trichechus manatus latirostris*), is naturally resistant to infectious diseases and its immune system is considered to be very well developed. Several of them kept in captivity developed distinct lesions which were diagnosed on their histological, ultrastructural and immunohistochemical features to be caused by papillomaviruses (Bossart *et al*. 2002). Preliminary immunological data plus daily observation of these captive manatees, showed that they were immunologically suppressed, and that the lesions could have been caused by activation of latent infections which were present three years earlier (Bossart *et al*. 2002).

Löhr *et al*. (2005) diagnosed sarcoids in a captive Grants zebra (*Equus burchellii boehmi*) in Mexico and a zebra from unknown taxon in Washington USA. Outbreaks of sarcoids in free-roaming Cape mountain zebras (*Equus zebra zebra*) in the Gariep Dam Nature Reserve, Free State Province, South Africa in 1995 (Nel *et al*. 2006) as well as the Bontebok National Park, Western Cape Province, South Africa in 1998 (Lange 2004) have been reported.

1.2.4 Diagnostic methods applicable to the study

1.2.4.1 Histopathological diagnosis

For light microscopy the formalin-fixed samples are embedded in paraffin wax and sections are cut from them using standard procedures, some of the latter are stained with haematoxylin and eosin and others with immunoperoxidase for immunohistochemical evaluation of bovine papillomavirus using the avidin-biotin technique. Formalin-fixed- or fresh samples of sarcoid tissue can be prepared for transmission electron microscopy (TEM). Examination of sections made from a typical lesion will reveal spherical papillomavirus particles 55-60 nm in diameter (Doane *et al*. 1987). The virus has a nonenveloped icosahedral structure which forms paracrystalline arrays in the nucleus of infected cells. Infected nuclei display numerous irregular clumps of condensed chromatin.

When examined under a light microscope specific features are observed in typical cases which consist of a dermal proliferation of fusiform- or spindle-shaped fibroblasts, forming whorls or interlacing bundles. In many cases the epidermis is absent but when it is present, and the lesion not ulcerated, it shows hyperplasia and hyperkeratosis as well as rete peg formation in most cases. At the dermo-epidermal junction, the characteristic "picket fence" appearance of the fibroblasts is present which is caused by their perpendicular orientation to the basement membrane (Ragland *et al*. 1970; Marti *et al*. 1993). Martens *et al*. (2000) could not distinguish clinical types of sarcoids according to their histology, and the increased numbers of dermal fibroblasts were the only common characteristic, the other changes being variable.

The virus can be demonstrated histopathologically (Haines & Chelack 1991) by using the avidin-biotin complex detection system. A polyclonal rabbit anti-papillomavirus antibody known to react with the L1 capsid proteins of most known papillomaviruses is used on formalin-fixed, paraffin-embedded sections of the lesions. The antibody used is that against a chemically disrupted virus.

As equine tissue is sometimes incorrectly diagnosed as pyogranulamatous dermatitis, fibropapilloma and fibrosarcoma, it has been suggested that molecular techniques would be more useful in the diagnosis than are histopathology (Angelos *et al*. 1991).

1.2.4.2 Molecular techniques

1.2.4.2.1 Blot-transfer hybridization

Blot-transfer hybridization methodology in conjunction with restriction enzyme endonuclease cleaving patterns of viral sequences has been used successfully to distinguish between the several different BPVs obtained from sarcoid lesions. Different enzyme cleaving patterns from those expected from the nucleotide sequence of the prototype viruses are sometimes obtained and it is thought that there might be variants of BPV which are specific for horses (Angelos *et al*. 1991).

1.2.4.2.2 *In situ* hybridization

In situ hybridization has been used by Lory *et al*. (1993) and Teifke *et al*. (1994) to detect BPV sequences in equine sarcoids, but the sensitivity of this technique is low. Bovine papillomavirus-DNA can be localized in large pleomorphic nuclei of the fibroblastic part of the tumours, mostly near the dermal-epidermal junction, but never in those of the epithelial cells.

1.2.4.2.3 Polymerase chain reaction

Polymerase chain reaction has been applied successfully to the investigation of BPV in equine sarcoids (Teifke & Weiss 1991). Up to 90% of sarcoid lesions from different animals were found to contain either BPV-1 or BPV-2. Bovine papillomavirus sequences were, however, only found in sarcoid lesions and not in any unaffected areas of the skin from these animals. The primers designed by Teifke & Weiss (1991) for the amplification of BPV-1 and -2 correspond to a region within the E5 open reading frame. The amplified product is 243 bp in length in BPV-1 and 246 bp in BPV-2. It was, however, necessary to do *Bst* X1 digestion of the amplified products to determine if the infection was BPV-1 or BPV-2 which is the reason why these primers were selected as the product from BPV-1 contains an endonuclease site that is absent in the BPV-2 genome (Bloch *et al*. 1994).

1.2.4.2.4 Real-time PCR

The LightCycler can be used with a wide variety of detecton formats, including: SYBR Green I, multicolour hybridization probes, monocolor simpleprobe probes, dual color hydrolysis probes and other techniques based on fluorescence resonance energy transfer. Real-time fluorescent hybridization probe analysis results in very high specificity. It is a valuable tool for reliable surveillance and routine testing of clinical specimens. This method has been successfully performed for the detection and differentiation of *Campylobacter jejuni* and *Campylobacter coli* (Abu-Halaweh *et al*. 2005), as well as Influenza A and B viruses (Smith *et al*. 2003) and the New World arenaviruses (Vieth *et al*. 2005), *Brucella* spp. (Al Dahouk *et al*. 2004) and the diagnosis of classical swine fever (Gaede 2002). Cubie *et al*. (2001) have shown that human papillomavirus (HPV)-16 and HPV-18 can be detected by rapid real-time PCR and can be differentiated by melting curve analysis. Other viruses, for instance the cytomegalovirus, has also been detected in plasma (Nitsche *et al*. 1999) by real-time PCR, and the ability to distinguish between herpes simplex virus 1 and 2 by melting curve analysis (Epsy *et al*. 2000), was also reported.

Type specific primers have to be designed as well as hybridization probes. These must be designed to anneal adjacent to each other on the same strand of product. The donor dye probe is labelled with fluorescein at the 3'-end and the acceptor dye probe with LightCycler Red at the 5' end. Only after hybridization are the two probes in close proximity, resulting in fluorescence resonance energy transfer between the fluorophores. The emitted fluorescence is measured at the respective wave length and is proportional to the amount of specific target sequences in the reaction mix. Each DNA fragment melts at a specific temperature, the melting temperature™, defined as the

temperature where 50% of the DNA is single stranded. The most important criteria that determine the Tm are the G + C content of the length of the fragment. The LightCycler instrument is able to monitor the fluorescence continuously, while raising the temperature gradually. When the temperature in the capillary reaches the Tm of the fragment under study, a sharp decrease in fluorescence results because one of the hybridization probes no longer binds with the target sequence.

Bogaert *et al*. (2006) selected a set of reliable reference genes for quantitative real-time PCR in normal skin and in equine sarcoids as the genes generally used can be involved in several functions and in tumours where the metabolism is eleviated the expression levels may vary considerably as well as in different tissues, cell types and disease stages which may lead to unreliable conclusions.

1.2.4.2.5 Single strand conformational polymorphism

Orita *et al*. (1989a) reported on a rapid and sensitive method that can detect sequence changes, including single-base substitutions as shifts in electrophoretic mobility. In this method the genomic DNA of a gene whose nucleotide sequences are known, was digested with restriction endonucleases, and denatured in an alkaline solution, and electrophoresis on a neutral polyacrylamide gel was performed. The results showed that a single–stranded DNA could take at least two different molecular shapes. They also demonstrated that the mobility shift due to a single base substitution of a single-stranded DNA in total digests of genomic DNA could be detected and was not influenced by the presence of a large amount of unrelated DNA fragments. Furthermore, families were tested and in each family the genotypes of the progenies were consistent with the parental genotypes, and therefore the observed single strand conformational polymorphisms (SSCP) are due to allelic variation of true Mendelian traits. Therefore, by neutral polyacrylamide gel electrophoresis single-stranded DNA fragments in which the nucleotide sequences differ at only one position, can be separated and can be used to locate genetic elements involved in hereditary diseases and to detect point mutations at various positions of the fragment. With this method Orita *et al*. (1989b) found that the most single base changes up to 200-base fragments could be detected as mobility shifts. The *Ras* oncogene was detected by this technique. Orita *et al*. (1989b) also demonstrated that *Alu* repeats in human are highly polymorphic.

Fujioka *et al*. (1995) performed an analysis of enterovirus genotypes using SSCP of PCR products. It demonstrated different electrophoretic profiles which proved to be useful for differentiating the genotypes of enteroviruses, and was useful for the rapid diagnosis of enteroviral infections.

Sheffield *et al*. (1993) used SSCP for mutations within different sized PCR products. They reported that the position of the base substitution is important in determining whether a mutation has been detected. The size of the fragment being analyzed dramatically influences the sensitivity of SSCP. They found that the optimal size of the fragment for sensitive base substitution is approximately 150 bp.

Hayashi (1991; 1992) considered PCR-SSCP analysis to be the most sensitive method for the detection of mutations based on PCR technology. The influence of temperature, the ratio of N_1N^1-methylenebisacrylamide to the concentration of total acrylamide monomer, and the concentration glycerol in the gel all play a role in the separation of mutated sequences. Other complications, for instance, smears or separation into more bands, even with the same sequence, can be overcome by running the electrophoresis under different conditions.

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CHAPTER TWO:

DETECTION OF BOVINE PAPILLOMAVIRUS DNA IN SARCOID-AFFECTED AND HEALTHY FREE-ROAMING ZEBRA (*Equus zebra*) POPULATIONS IN SOUTH AFRICA

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ABSTRACT

The endangered Cape mountain zebra (*Equus zebra zebra*) is protected in small numbers in a few isolated populations in South African game parks. Since 1995, sarcoid lesions appeared in zebras in two of the parks. This study was undertaken to investigate if bovine papillomavirus (BPV) is associated with sarcoids in these zebras. A conventional PCR, targeting the E5 ORF of BPV, and subsequent RFLP analysis were initially used to demonstrate the presence of BPV-1 and -2 DNA in zebra sarcoid tumours. A rapid, sensitive and reliable real-time PCR to detect and distinguish between BPV-1 and -2 infections in zebras was then developed. With this assay it was demonstrated that BPV-1 and -2 DNA (either single or mixed infections) are present in sarcoid tumour, healthy skin and blood of sarcoid-affected and healthy zebras from sarcoid-affected parks as well as in the blood of zebras from parks where no sarcoid has been observed before.

Keywords: Cape mountain zebra; bovine papillomavirus; sarcoid; real-time PCR

2.1 INTRODUCTION

The Cape mountain zebra (*Equus zebra zebra*) is one of the rarest mammals in the world (Penzhorn 2003) and classified as endangered on the International Union for the Conservation of Nature and Natural Resources (IUCN) Red List 2006. It is protected in small numbers in a few isolated populations in South African game parks in their natural habitat. Of the three extant species of zebra in the family *Equidae*, genus *Equus*, subgenus *Equus zebra*, consists of two subspecies: the Cape mountain zebra, *Equus zebra zebra*, from the Eastern and Western Cape Province, South Africa and Hartmann's mountain zebra, *Equus zebra hartmannae* of Namibia and Angola. Cape mountain zebra populations have historically declined due to hunting, habitat loss, droughts and the interbreeding of the two subspecies. Although their numbers have slowly increased in recent years, the genetic diversity and the small numbers of Cape mountain zebras are of great concern as the uneven distribution of a few relatively large populations makes them still vulnerable (Novellie *et al*. 2002).

Zebras respond differently to most infectious diseases when compared to horses. They are resistant to African horsesickness (Barnard 1993, 1994; Lord *et al*. 1997), a viral disease of horses. Equine herpesvirus (EHV) infections appear to be widespread in free-ranging zebras, they may be a natural host for EHV, although there is no evidence of mortality due to EHV. (Borchers & Fröhlich 1997; Blunden *et al*. 1998; Borchers *et al*. 2006). The presence of antibodies against equine encephalosis virus (EEV) has also been shown (Barnard & Paweska 1993). Although no evidence of infection with equine arteritis virus could be found in South Africa (Barnard & Paweska 1993), antibodies were demonstrated in Burchell's zebras (*Equus burchelli*) from the Serengeti ecosystem (Borchers *et al*. 2005). Bovine papillomaviruses (BPV) are associated with sarcoids, the most common dermatological skin lesion in equidae (Jackson 1936; Goodrich *et al*. 1998). Bovine papillomavirus types 1 and 2 (BPV-1 and BPV-2) have been detected in sarcoid tumours of horses, donkeys and mules (Lancaster *et al*. 1977; Angelos *et al*. 1991; Bloch *et al*. 1994; Reid *et al*. 1994; Nasir *et al*. 1997 Nasir & Reid 1999; Chambers *et al*. 2003b) and two cases were reported from captive zebras (*Equus burchelli boehmi*), one from Mexico and one from a private wild animal farm in Washington State, United States of America (Löhr *et al*. 2005). In 1995, sarcoid lesions appeared in Cape mountain zebras in the Gariep Dam Nature Reserve, Free State Province (Nel *et al*. 2006) and in 1998 in the Bontebok National Park, Western Cape Province (Lange 2004). An isolated case of a zebra that was euthanized due to the severity of sarcoid lesions was reported in 2004 in the Mountain Zebra National Park, Eastern Cape Province. Subsequently four more cases were reported (personal communication: Dr Dave Zimmerman). Sarcoid lesions are the most common

dermatological skin lesion in domestic equidae (Goodrich *et al*. 1998; Jackson 1936). The term "equine sarcoid" was first used by Jackson in South Africa in 1936 to describe a distinctive fibroblastic neoplasm occurring in the skin of horses, donkeys and mules (Jackson 1936). It was also used to distinguish the neoplasm from papilloma, fibroma and fibrosarcoma. Equine sarcoid is a locally aggressive, non-regressing, fibroblastic skin tumour, which does not produce infectious virions (Amtmann *et al*. 1980; Lancaster 1981).

All papillomaviruses are classified in the family *Papillomaviridae* (Bernard 2006). This large family of animal and human viruses generally infect epithelial cells causing hyperproliferative lesions known as warts, papillomas or condylomas. Typically these lesions are benign, self-limiting and spontaneously regress, although some are linked to malignancy such as the human papillomavirus 16 and 18 (WHO / IARC 1995). Some types of papillomaviruses can also infect fibroblasts and induce fibro-epithelial tumours, including BPV-1 and -2, which cause benign fibropapillomas in cattle (Nasir *et al*. 2007). All papillomaviruses except one are strictly species-specific, only known exception being naturally occurring cross-species infection of horses, donkeys and mules with BPV-1 and -2 (Nasir *et al*. 2007) in which they are associated with sarcoids (Lancaster & Olson 1980; Nasir & Reid 1999; Campo 2002).

Both BPV-1 and BPV-2 have a genome of 7 900 bp of double stranded DNA and are composed of early (E) and late (L) genes which can be divided into several open reading frames: viral replication (E1), regulation of transcription (E2), coding for cytoplasmic proteins (E4), transforming proteins (E5, E6 and E7) as the early genes and L1 and L2 coding for capsid proteins as late genes (Chambers *et al*. 2003a; Nasir & Campo 2008). Studies concerning gene expression have largely focused on expression of the major BPV early genes, E2, E5, E6 and E7 (Bogaert *et al*. 2007; Yuan *et al*. 2007).

Equine sarcoids appear in different clinical entities and can be classified into six distinct clinical types (Knottenbelt 2005). Histopathological examination is often required to confirm diagnosis and to distinguish them from other skin lesions (Jackson 1936; Goodrich *et al*. 1998; Martens *et al*. 2000). Polymerase chain reaction techniques are suitable for the investigation of papillomavirus-associated benign and malignant lesions (Bloch *et al*. 1994) and PCR has been used to elucidate the role of BPV in the induction of equine sarcoids. Most equine sarcoids are found to contain viral sequences of either BPV-1 or BPV-2 (Ragland *et al*. 1970; Amtmann *et al*. 1980; Lancaster & Olson 1980; Lancaster 1981; Angelos *et al*. 1991; Teifke & Weiss 1991; Reid & Smith 1992; Otten *et al*. 1993; Reid *et al*. 1994; Trendfield *et al*. 1985; Carr *et al*. 2001a, 2001b; Bogaert *et al*. 2005;

Yuan *et al*. 2007). Several workers have used primers specific for the amplification of a 244 bp and 247 bp region of the E5 open reading frame (ORF) of BPV-1 and -2, respectively (Otten *et al*. 1993; Bloch *et al*. 1994; Teifke *et al*. 1994; Carr *et al*. 2001b; Martens *et al*. 2001). Restriction fragment length polymorphism (RFLP) is then generally used to differentiate between BPV-1 and -2 as the BPV-1 amplified product contains a *Bst*X1 restriction site which is absent in BPV-2 (Teifke & Weiss 1991; Bloch *et al*. 1994). The PCR assay has been successfully used to demonstrate BPV DNA in up to 100% of examined equine sarcoids in several studies (Otten *et al*. 1993; Teifke *et al*. 1994; Carr *et al*. 2001b; Martens *et al*. 2001;). Recently, quantitative real-time PCR assays have been developed and used to determine viral load and the expression of BPV E2, E5, E6 and E7 genes in equine sarcoids and inflammatory skin conditions (Bogaert *et al*. 2007; Yuan *et al*. 2007).

The purpose of this study was to determine if BPV is also present in sarcoid in free-roaming zebras by using PCR and RFLP analysis, and to develop a real-time PCR diagnostic method to detect and distinguish between BPV-1 and -2.

2.2 MATERIALS AND METHODS

2.2.1 Study population and sample collection

Samples were taken from a total of 149 zebras located in different national parks in South Africa (**Figure 4**). The latter can be divided into parks where sarcoid tumours have been observed and those where they have not been observed. Sarcoid tumour samples included in this study were obtained from Cape mountain zebras (*E. zebra zebra*) from the Gariep Dam Nature Reserve, Free State Province (n=9), Bontebok National Park, Western Cape Province (n=2) and Mountain Zebra National Park, Eastern Cape Province (n=1). In most instances, healthy skin and blood samples were also collected from the sarcoid-affected zebras (**Table 1**). Additionally, blood samples were collected from healthy, sarcoid-unaffected zebras (n=51) in these parks. In the case of the Bontebok National Park, blood was also collected from seven zebras that had previously suffered from sarcoids, but had been successfully treated surgically and subsequently treated by parenteral administration of fluouracil or an autogenous vaccine (Lange 2004).

In parks where no sarcoid tumours had previously been observed, blood samples were collected from Hartmann's mountain zebras (*Equus zebra hartmannae*) in the Augrabies National Park, Northern Cape Province (n=25) and the Cederberg Wilderness Area, Western Cape Province (n=8), and Burchell's zebras (*Equus burchelli*) (n=17) and Cape mountain zebras (*E. zebra zebra*) (n=36) in the Karoo National Park, Western Cape Province.

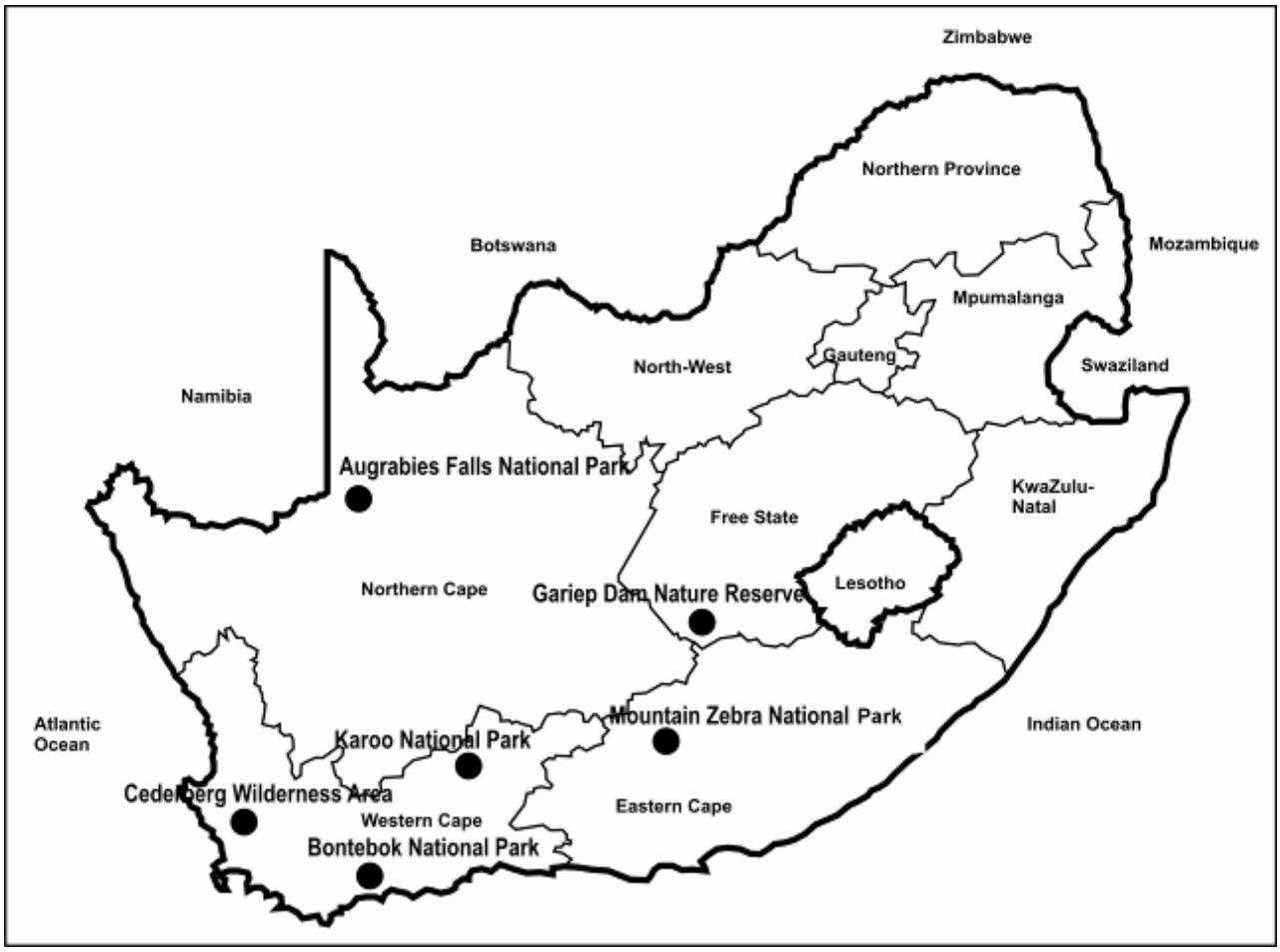

Figure 4 Map of South Africa illustrating the different areas where zebras have been sampled

Sarcoid tumours, which occurred in most instances ventrally, and healthy skin samples were collected by means of a biopsy punch, which excised a piece of tissue 10 x 3 x 3 mm in size. The healthy skin samples were taken at a distance of at least 60 cm away from the sarcoid tumour. These were stored in sterile tubes at 4 °C until further analysis. Duplicate sarcoid samples were stored in a 10% dilution of formaldehyde. Blood samples were collected in EDTA-buffered tubes by venipuncture of the jugular vein and stored at 4 °C.

Tissue blocks of a histopathologically confirmed equine sarcoid from a horse (n=1) and a bovine papilloma (n=1) were obtained from private veterinarians and were included in the study to serve as positive control samples. Autogenous vaccines comprising formalin-inactivated, cell-free supernatant fluid prepared from finely minced sarcoid tumours obtained from the Gariep Dam Nature Reserve and Bontebok National Park were also included in the study (Personal communication Dr A L Lange). Blood from sarcoid-free zebras from areas where no sarcoid occurred acted as negative controls.

2.2.2 Histopathology

The formalin-fixed samples were dehydrated and paraffin wax-embedded for routine histological processing. All tumour sections were stained with haematoxylin and eosin for light microscopical study.

Table 1 Summary of samples collected.

Zebra species	National Park / Game Reserve	Number	Samples collected		
			Sarcoid tumour	Healthy skin	Blood
	Sarcoid-affected parks				
Cape Mountain zebra (*Equus zebra zebra*)	Gariep Dam Nature Reserve (Free State Province)	Affected (9)	9	9	9
		Healthy (19)	NA	NC	19
	Bontebok National Park (Western Cape Province)	Affected (2)	2	NC	2
		Treated* (7)	NA	NC	7
		Healthy (13)	NA	NC	13
	Mountain Zebra National Park (Eastern Cape Province)	Affected (1)	1	NC	1
		Healthy (12)	NA	NC	12
Total number of samples collected			***12***	***9***	***63***
	Sarcoid-unaffected parks				
Cape mountain zebra (*Equus zebra zebra*)	Karoo National Park (Western Cape Province)	36	NA	NC	36
Burchell's zebra (*Equus burchelli*)	Karoo National Park (Western Cape Province)	17	NA	NC	17
Hartmann's zebra (*Equus zebra hartmannae*)	Augrabies National Park (Northern Cape Province)	25	NA	NC	25
	Cederberg Wilderness Area (Western Cape Province)	8	NA	NC	8
Total number of samples collected			***NA***	***NC***	***86***

NA = Not applicable
NC = Not collected
* Zebra that had suffered from sarcoid lesions before, but which were successfully treated with surgery

2.2.3 DNA extraction

DNA was extracted from 200 µl of blood, vaccine or 25 mg of tissue (healthy skin or sarcoid) 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.

2.2.4 Conventional PCR amplification of a region of the E5 open reading frame

PCR was used for the detection of BPV DNA in blood (n=51), sarcoid tumour (n=12) and skin samples (n=9). The primers used were those designed by Teifke and Weiss (1991), for the amplification of a region of the E5 ORF of both BPV-1 and BPV-2 (forward primer: 5'-CAA AGG CAA GAC TTT CTG AAA CAT-3', and reverse primer: 5'-AGA CCT GTA CAG GAG CAC TCA A-3'). The amplification mixture consisted of 2.5 µl DNA (~75 ng), 12.5 µl Sigma REDTaq™ ReadyMix PCR Reaction Mix (Sigma®, California, USA), 0.1 µM of each primer and nuclease-free water to a total volume of 25 µl. Amplification was performed in an automated thermocycler (Perkin-Elmer, Foster City, CA). Initial denaturation was performed at 94 °C for 10 minutes. This was followed by 35 cycles, each consisting of denaturation at 94 °C for 15 seconds; annealing at 60 °C for 30 seconds and extension at 72 °C for 30 seconds. Final extension was at 72 °C for 5 minutes before cooling down to 4 °C. The amplified DNA products were electrophoresed using a 2% agarose gel, stained with ethidium bromide and photographed.

Differentiation between BPV-1 and -2 was performed using the restriction endonuclease *Bst*XI on the E5 ORF amplification products (Bloch *et al*. 1994; Teifke & Weiss 1991). Twelve samples were examined for the presence of a *Bst*X1 site in the amplified product. The restriction enzyme digest mixture consisted of 2 µl 10x restriction buffer, 2 µg acetylated BSA, 5 U *Bst*X1 restriction enzyme (Promega, Madison, USA), 2.5 µg amplified DNA product and nuclease-free water to a final volume of 20 µl. The digestion mixture was incubated at 37 °C for 4 hours. The resultant restriction products were separated by electrophoresis using a 6% polyacrylamide gel, stained with ethidium bromide and visualized by UV trans-illumination.

2.2.5 Real-time PCR

A hybridization probe real-time PCR assay was developed for the detection and differentiation of BPV-1 and -2 DNA in blood (n=149) sarcoid tumour (n=12) and skin samples (n=9).

2.2.5.1 Primer and hybridization probe design

The conventional PCR primer set as described above (Teifke & Weiss 1991) was used for the amplification of a 244 and 247 bp region of the E5 ORF of BPV-1 and -2, respectively. For the specific detection of BPV-1 amplicons, hybridization probes BPV-1 anchor (5'-ACT GG TGT ACT ATG CCA AAT CTA TGG TTT CTA TTG-Fluor-3') and BPV-1 sensor (5'-LC640-CTT GGG ACT AGT TGC TGC AAT GCA ACT-Pho-3') were designed complementary to a BPV-1 specific region within the amplicon using the LightCycler® Probe Design Software 2.0. For the specific detection of BPV-2 amplicons, hybridization probes BPV-2 anchor (5'-TTT AAT CAC TGC CAT TTG TTT TTT TCA TAT CTC GT-Fluor-3') and BPV-2 sensor (5'-LC705-AGG CAT ACT ATG CCG AAT CTA TGG TTT CTA TTG TT-Pho-3') were designed. All primers and fluorescently-labeled hybridization probes were synthesized by Metabion International AG (Martinsried, Germany).

2.2.5.2 Real-time PCR conditions

The amplification mixture consisted of 2 µl of 10x LightCycler® FastStart DNA Master Hybridization Probes mix (Roche Diagnostics, Mannheim, Germany), 3 mM $MgCl_2$, 0.5 µM of each primer, 0.2 µM of each hybridization probe, 1 U uracil deoxy-glycosylase (UDG) (Roche Diagnostics, Mannheim, Germany) and 2 µl (~30 ng) of template DNA to a final volume of 20 µl. Temperature cycling was performed in a LightCycler® v2.0 (Roche Diagnostics, Mannheim, Germany). The UDG was activated at 40 °C for 10 minutes, followed by activation of the FastStart *Taq* DNA polymerase at 95 °C for 10 minutes. Forty-five cycles of denaturation at 95 °C for 10 seconds, annealing at 60 °C for 10 seconds, and signal acquisition and extension at 72 °C for 20 seconds. After amplification, a melting curve was generated by heating the amplicons from 40 °C to 95 °C with a heating rate of 0.2 °C /second. Fluorescence values were measured at 640 and 705 nm and the results analyzed with the Roche LightCycler® Software v4.0. The presence of a single amplification product was verified on a 2% agarose gel.

2.2.5.3 Quantitative sensitivity

To generate a standard curve for quantitative BPV real-time PCR, a 637 bp DNA fragment of the E5 ORF of vaccine sample 124 was amplified, cloned and subjected to real-time PCR. Previously, real-time PCR indicated that this sample was infected with both BPV-1 and -2.

The newly designed primer pair F_3610 (5'-GCT AAC CAG GTA AAG TGC TAT C-3') and R_4247 (5'-TGC TTG CAT GTC CTG TAC AGG T-3') were used for amplification of the 637 bp DNA fragment. The amplification mixture contained 2.5 µl DNA (~75 ng), 12.5 µl Expand High Fidelity PCR Master (Roche Diagnostics, Mannheim, Germany), 0.1 µM of each primer and nuclease-free water to a total volume of 25 µl. Thermal cycling conditions were the same as described in 2.2.5.2. The obtained amplicon was purified prior to cloning using the QIAquick PCR Purification Kit (QIAGEN, Southern Cross Biotechnologies) following to the specifications of the manufacturer. The purified amplicon was 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 using the High Pure Plasmid Purification Kit (Roche Diagnostics, Mannheim, Germany). The purity and concentration of plasmid DNA (clone 124/12, representing BPV-1) were determined by optical density measurements. A plasmid size of 3.015 kb was used to calculate the concentrations in plasmid copies per microliter corresponding to BPV E5 ORF equivalents. To test the sensitivity of the real-time PCR, a 10-fold dilution series was prepared, subjected to real-time PCR and a standard curve was generated using the LightCycler™ software (Roche Diagnostics, Mannheim, Germany).

2.2.6 Molecular cloning and sequencing

Since double peaks were observed in the melting temperature curves generated from both BPV-1 and -2 hybridization probe sets, the complete E5 ORF of five sarcoid tumour samples collected from the Cape mountain zebras in the Gariep Dam Nature Reserve, as well as the histopathologically-confirmed equine sarcoid and bovine papilloma, was amplified, cloned and sequenced to confirm that the amplicons obtained were indeed related to BPV. The primer pair F_3610 and R_4247 was used as described above to amplify a 637 bp region of both BPV-1 and -2. The amplicons were purified, cloned into the pGEM®-T vector (Promega pGEM–T Easy Vector System, Promega, Madison, USA), and transformed into competent JM 109 *E. coli* cells (Promega, Madison, USA) as described above. 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) and aligned with published sequences of related genera using ClustalX (version 1.81 for Windows). A BLAST search was performed using the Blastn algorithm.

2.3 RESULTS

2.3.1 Conventional PCR amplification of a region of the E5 ORF

All tumours collected (n=12) exhibited similar histological changes, including dermal proliferation of spindle shaped fibroblasts forming whorls, epidermal hyperplasia and hyperkeratosis and rete peg formation typical of sarcoid (Jackson 1936; Martens *et al*. 2000).

Conventional PCR (Teifke & Weiss 1991) was used for the detection of BPV DNA in the Cape mountain zebras in the Gariep Dam Nature Reserve, Bontebok National Park and Mountain Zebra National Park. Sarcoid tumour (n=12), healthy skin (n=9) and blood (n=51) samples were analyzed. All 12 sarcoid tumour samples analyzed resulted in the expected amplification product of ~244 bp for BPV-1 and/or ~247 bp for BPV-2 and were subsequently subjected to *Bst*X1 restriction digestion for discrimination. No amplification product could be detected in the healthy skin or blood of these animals.

2.3.2 *Bst*X1 restriction digestion of the PCR amplified E5 ORF

Restriction fragment length polymorphism (RFLP) was used to differentiate between the amplified E5 ORF of BPV-1 and -2 (**Figure 5**). The 244 bp BPV-1 E5 ORF amplified product has a *Bst*X1 restriction site resulting in two fragments of 130 bp and 114 bp after restriction digestion. The BPV-2 amplified product does not contain a *Bst*X1 recognition sequence (Bloch *et al*. 1994; Teifke & Weiss 1991). The digestion of the amplified E5 ORF of the 12 sarcoid tumour samples investigated revealed that seven (58%) were derived from BPV-1 infection, four (33%) from BPV-2 and one tumour (9%) had both BPV-1 and -2 DNA present (**Table 2**). The autogenous vaccine from (124) from Bontebok National Park contained both BPV-1 and -2 (**Figure 5**).

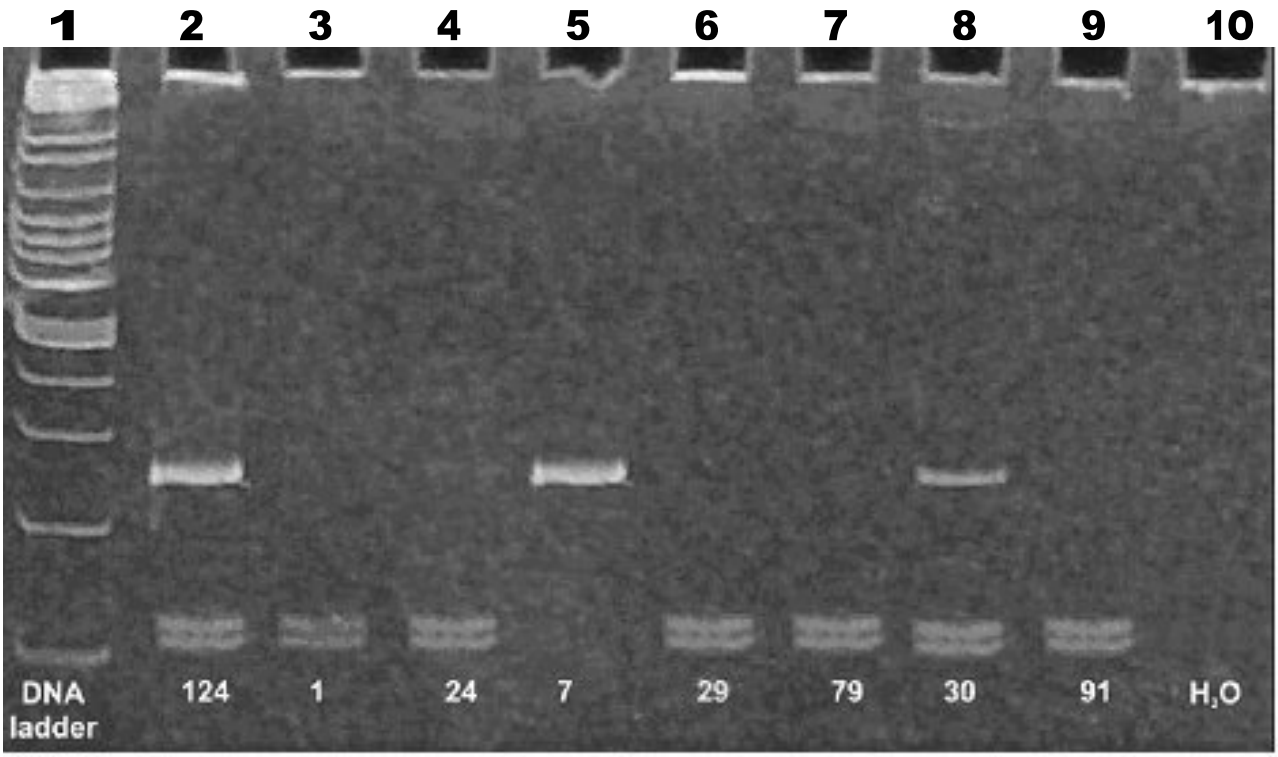

Figure 5 Polyacrylamide gel showing *Bst*XI restriction enzyme digestion of the PCR amplified E5 ORF of zebra sarcoid tumours. Lane 1=100 bp ladder, Lane 2 & 8=samples demonstrating both BPV-1 and BPV-2 infection, Lane 3, 4, 6, 7, 9=BPV1 infection, Lane 5=BPV 2 infection. Lane 10=negative control.

Table 2 Summary of results obtained by *Bst*X1 restriction digestion and real-time PCR.

			Sarcoid tumour		Healthy skin		Blood	
Zebras	**National Park / Game Reserve**	**Number**	**RFLP**	**Real-time PCR**	**RFLP**	**Real-time PCR**	**RFLP**	**Real-time PCR**
	Sarcoid-affected parks							
Cape Mountain zebra	Gariep Dam Nature Reserve	Affected (9)	I=4 II=4 I&II=1	I=4 II=4 I&II=1		I=3 II=1 I&II=5		I=5 II=0 I&II=2
		Healthy (19)						I=7 II=3
	Bontebok National Park	Affected (2)	I=2	I=2				I&II=1
		Treated (7)						I=4 II=1
		Healthy (13)						I=5 II=2
	Mountain Zebra National Park	Affected (1)	I=1	I=1				I=1
		Healthy (12)						I=1 II=1
Summary of BPV type detected			***I=58% II=33% I&II=9%***	***I=58% II=33% I&II=9%***		***I=33% II=11% I&II=56%***		***I=36% II=11% I&II=5% None=48%***
	Sarcoid unaffected parks							
Cape mountain zebra	Karoo National Park	36						I=10 II=3
Burchell's zebra	Karoo National Park	17						I=8 II=2
Hartmann's zebra	Augrabies National Park	25						I=8 II=2
	Cederberg Wilderness Area	8						I=2
Summary of BPV type detected								***I=33% II=8% I&II=0% None=59%***

2.3.3 Real-time PCR

A hybridization probe real-time PCR assay was developed for the detection and differentiation of BPV-1 and -2 DNA. The primers amplified a 244 bp or 247 bp region of the E5 ORF of BPV-1 and respectively. Two separate hybridization probes sets were designed and used in a multiplex format for the specific detection of BPV-1 and -2 DNA.

2.3.3.1 Specificity of the real-time PCR assay

The specificity of the real-time PCR assay was investigated by testing the PCR-confirmed sarcoid tumour samples from Cape mountain zebras from the Gariep Dam Nature Reserve (n=9), Bontebok National Park (n=2) and Mountain Zebra National Park (n=1). Specimens from the histopathologically confirmed equine sarcoid from the horse (n=1) and bovine papilloma (n=1) were also included as positive controls. Real-time PCR amplicons obtained from representative samples were directly sequenced and a BLAST search confirmed the presence of BPV DNA in all specimens.

For the specific detection of BPV-1 DNA, an increase in fluorescence was detected at 640 nm. However, sarcoid tumour samples containing BPV-2 DNA, as determined with *Bst*X1 restriction digests, also showed a slight increase in fluorescence at 640 nm (**Figure 6a**). This indicated a possible cross-reaction with the probe. Melting curve analysis indicated two BPV-1-specific melting peaks, at 62.90 ±1.24 °C and 68.17 ±0.71 °C (**Figure 6b**). Both melting peaks were consistently observed. BPV-2 DNA gave a melting peak at 58.48 ±0.61 °C confirming cross-reaction with the probe. No fluorescence was observed in the negative control.

For the specific detection of BPV-2 an increase in fluorescence at 705 nm was observed. Amplification curves were not as smooth as expected (**Figure 7a**). Melting curve analysis indicated two BPV-2-specific peaks at 58.86 ±0.60 °C and 64.06 ±0.59 °C (**Figure 7b**). BPV-1 DNA showed cross reaction with the probe with a melting peak at 46.25 ±1.22 °C. These were consistently observed. No fluorescence was observed in the negative control.

The histopathologically confirmed sarcoid from the horse and the bovine papilloma control samples were infected with BPV-1. Of the 12 sarcoid tumour samples investigated, seven (58%) contained BPV-1 and four (33%) BPV-2 (**Table 2**) (**Figure 8**). One tumour (9%) had both BPV-1 and -2 DNA present as was evident from the melting curve profile (**Figure 6c**). These results are in concordance with the results obtained with *Bst*X1 restriction digests.

2.3.3.2 Sensitivity of the real-time PCR assay

To generate a standard curve for quantitative BPV real-time PCR, a 637 bp DNA fragment of the E5 ORF was cloned, a 10-fold dilution series was prepared, and subjected to real-time PCR. Fluorescence was detected up to an average threshold value (Ct) of 36.61 for the highest solution (10^{-8}). A standard curve was generated using the LightCyclerTM software. The assay had an amplification efficiency of 2.058. An amplification efficiency of 2.00 is considered ideal, and corresponds to a doubling of copy number for every PCR cycle (Ulrich *et al*. 2006). The BPV assay could reliably amplify and detect BPV DNA up to a dilution of 2 x 10^{-17}g of plasmid, corresponding to the amplification of five genome copies per reaction.

2.3.3.3 Detection of BPV DNA in clinical samples

DNA obtained from healthy skin biopsies of nine sarcoid-affected zebra from Gariep Dam Nature Reserve tested positive for the presence of BPV DNA using the real-time PCR assay (**Table 2**). Of the nine samples tested, 33% were positive BPV-1, 11% foe BPV-2 and 56% had both BPV-1 and -2 DNA present. In addition, the real-time PCR assay was used for the detection or absence of BPV DNA in blood samples of the sarcoid-affected (n=12) and –unaffected (n=51) Cape mountain zebras, as well as those from Cape mountain zebras (n=36), Hartmann's mountain zebras (n=33) and Burchell's zebras (n=17), from parks in which no sarcoid tumours have previously been observed (**Table 2**). Of the 12 sarcoid-affected Cape mountain zebras, the blood of six (50%) was infected with BPV-1. None of the latter were infected with BPV-2 alone, but three (25%) were infected with both BPV-1 and -2 (**Figure 9**). Three (25%) of the blood samples showed no evidence of infection. Of the sarcoid-unaffected Cape mountain zebras, in the affected parks, the blood of 17 (33%) was infected with BPV-1 and seven (14%) revealed evidence of a BPV-2 infection. These included the seven zebras that had recovered from sarcoid after treatment, four of which were infected with BPV-1 and one with BPV-2. A total of 27 (53%) of the 51 blood samples collected from unaffected zebras in the affected parks showed no infection. Skin samples (n=9) of the Cape mountain zebras in the Gariep Dam Nature Reserve were also subjected to the real-time PCR assay. Three (33%) of the samples were infected with BPV-1, one (11%) with BPV-2 and five (56%) with both BPV-1 and -2.

Analysis of the blood from the zebras located in parks where no sarcoid tumours had previously been observed showed that 10 (28%) of the Cape mountain zebras had BPV-1 and three (8%) had BPV-2 infection; 10 (30%) of the Hartmann's mountain zebras were infected with BPV-1, and two (6%) had BPV-2 infections, eight (47%) of the Burchell's zebras were infected with BPV-1 and -2

(12%) had BPV-2 infection (**Figure 9**). No mixed infections were observed in any of these samples. A total of 51 (59%) of the blood samples from the unaffected parks showed no evidence of infection.

The autogenous vaccine which was used in the treatment of sarcoid infected Cape mountain zebras in the Bontebok National Park and Gariep Dam Nature Reserve was also subjected to the real-time PCR assay. The results revealed that the vaccine prepared from tumour of zebras from the Bontebok National Park was infected with both BPV-1 and -2 while those from the Gariep Dam Nature Reserve was infected with only BPV-1.

2.3.4 Molecular cloning and sequencing

Since double peaks were observed in the melting temperature curves generated from both BPV-1 and -2 hybridization probe sets, a 637bp fragment of the E5 ORF of the equine sarcoid and bovine papilloma positive control samples, five sarcoid tumour samples from Cape mountain zebra from the Gariep Dam Nature Reserve were amplified, cloned and sequenced to confirm that the amplicons obtained were indeed related to BPV. A BLAST search was performed and revealed that both the equine sarcoid and bovine papilloma positive control samples, as well as three of the sarcoid tumour samples and the Gariep Dam Nature Reserve vaccine, showed the highest similarity (~98%) with the BPV-1 subtype IV E5 protein encoding gene (accession number AY232260). Two sarcoid tumour samples showed highest similarity (~98%) with BPV type 2 E5 protein encoding gene (M20219). The vaccine from the Bontebok National Park was confirmed to be infected with both BPV-1 and -2. The BPV-2 clone showed the highest similarity (~99%) with the BPV-2 E5 protein encoding gene (M20219 and AY232264). The obtained sequences were deposited in GenBank under accession numbers FJ648519 to FJ648528.

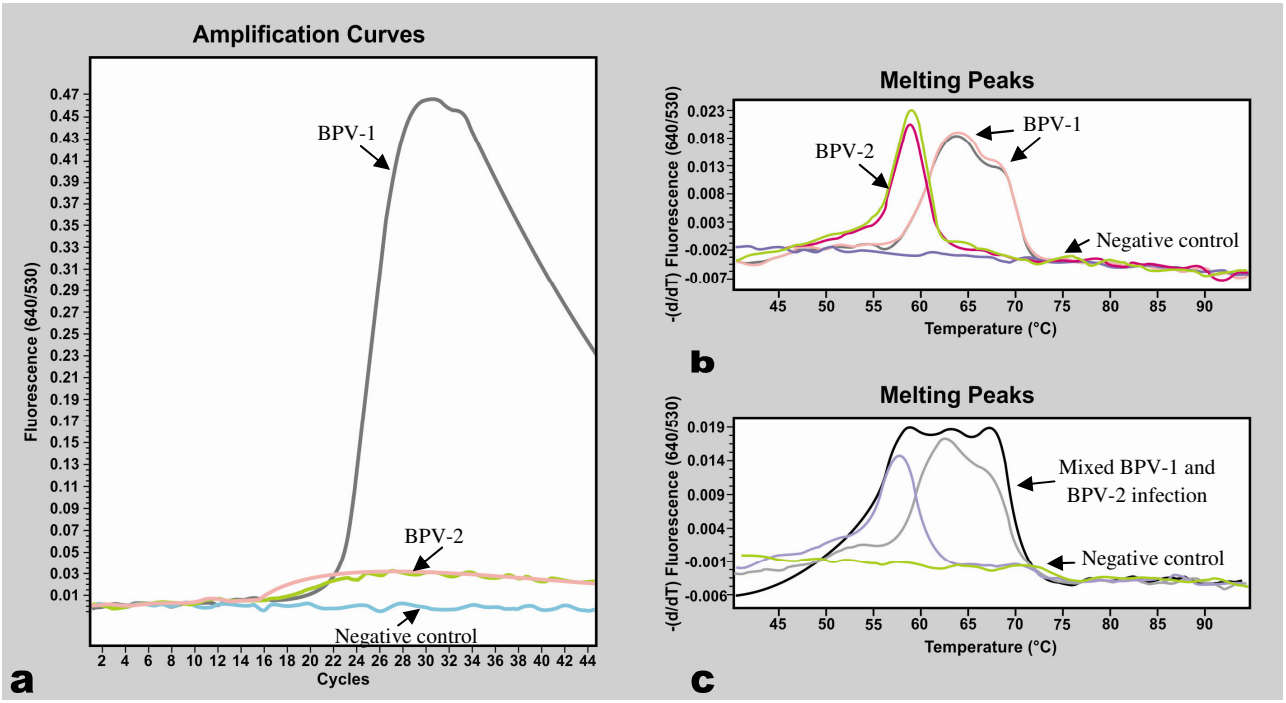

Figure 6 Real-time PCR results for the detection of BPV-1 DNA. (a) Amplification curves showing the increase in fluorescence at 640 nm. (b) Melting curve analysis of the amplicons generated from BPV-1 and BPV-2 positive DNA. Two peaks were obtained for BPV-1 DNA, at 62.90 ±1.24 °C and 68.17 ±0.71 °C. The BPV-2 DNA melting peak was at 58.48 ±0.61 °C. No fluorescence was observed in the negative control. (c) Melting curve analysis of amplicons generated by mixed BPV-1 and BPV-2 infection.

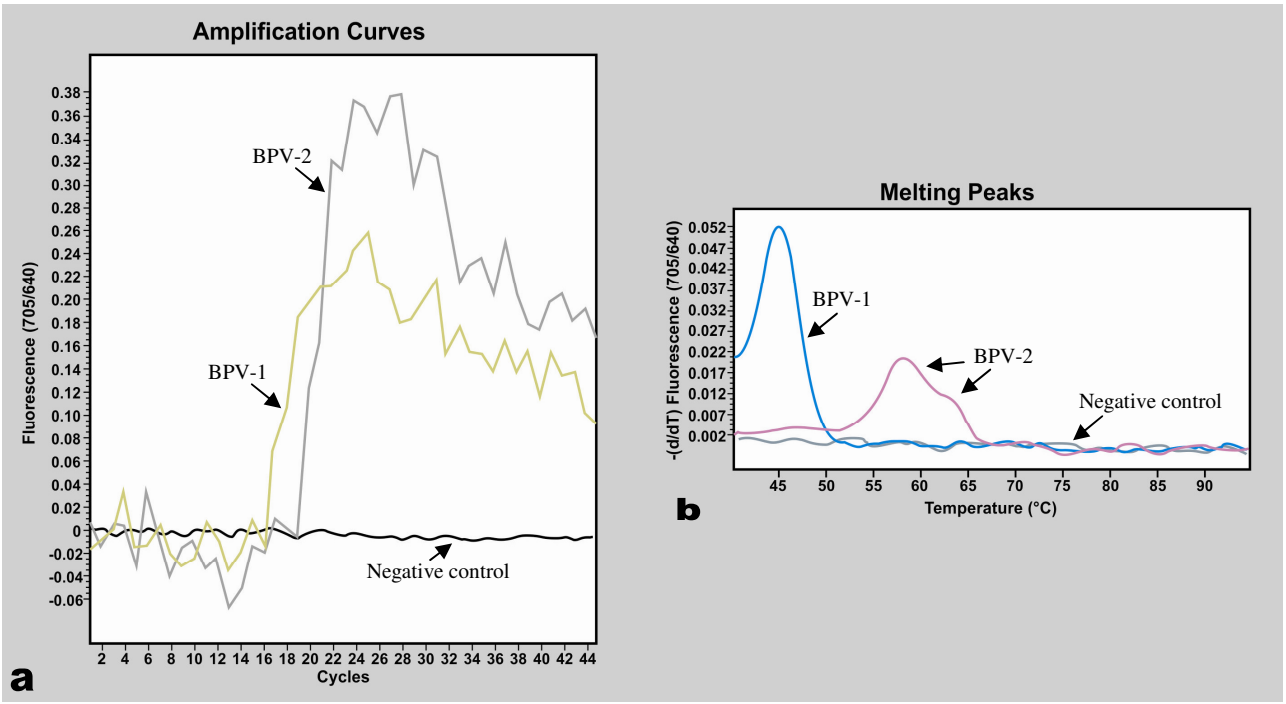

Figure 7 Real-time PCR results for the detection of BPV-2 DNA. (a) Amplification curves showing the increase in fluorescence at 705 nm. (b) Melting curve analysis of BPV amplicons. Two peaks were obtained for BPV-2 DNA, at 58.86 ±0.60 °C and 64.06 ±0.59 °C. The melting peak for BPV-1 DNA was at 46.25 ±1.22 °C. No fluorescence was observed in the negative control.

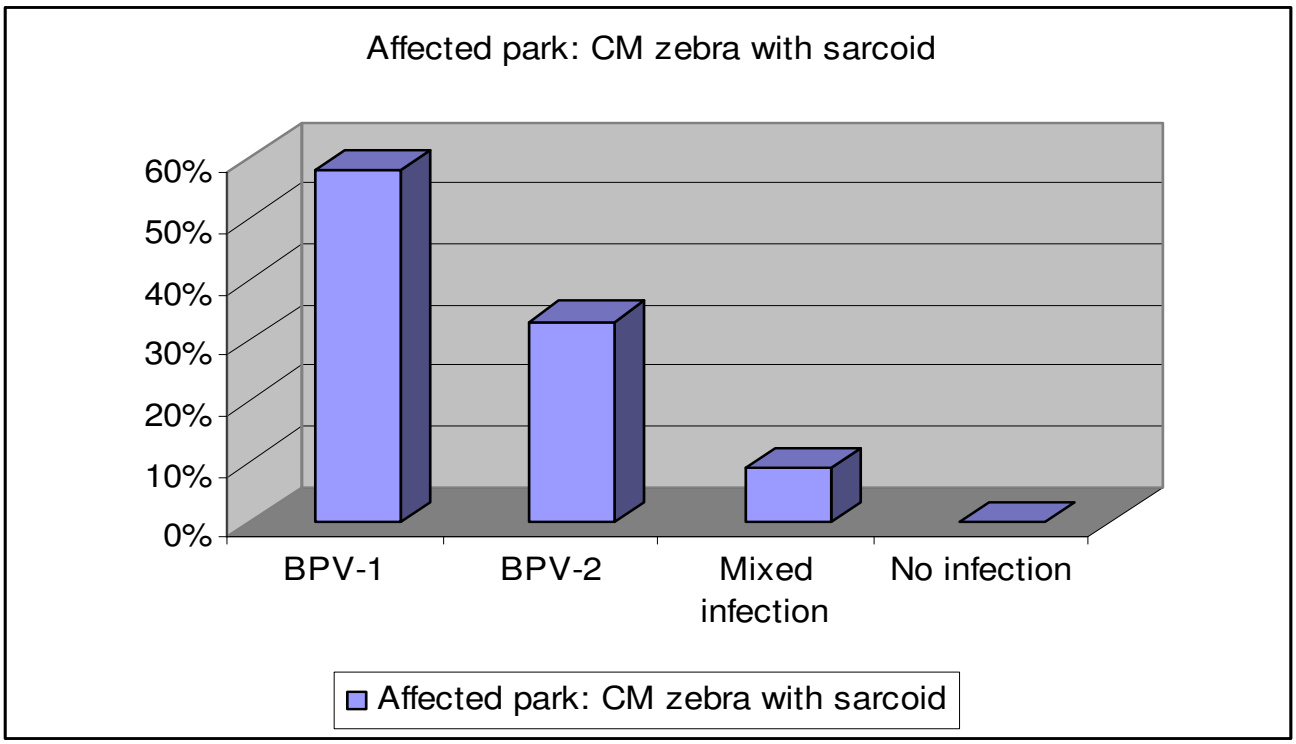

Figure 8 Prevalence of BPV infection in zebra sarcoid tumour samples collected from the Gariep Dam Nature Reserve, Bontebok National Park and Mountain Zebra National Park as determined with real-time PCR.

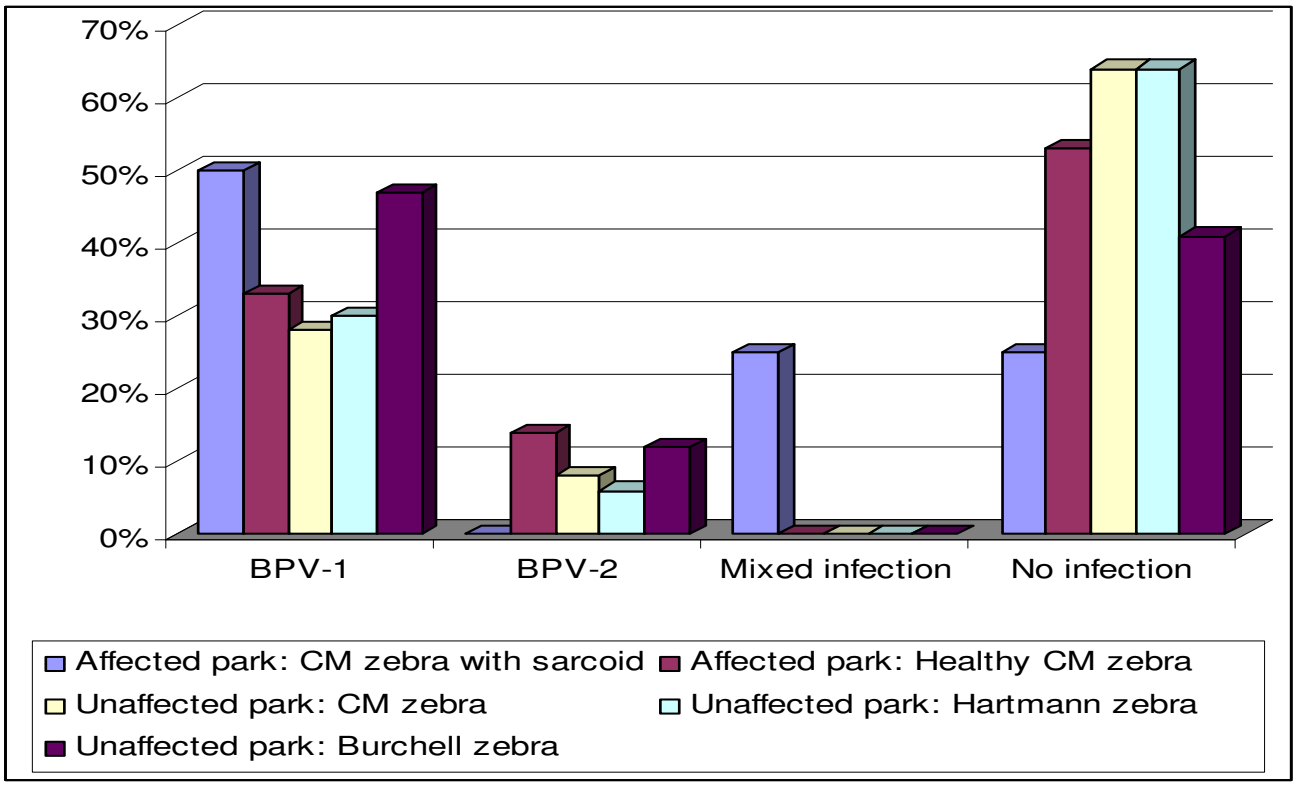

Figure 9 Prevalence of BPV infection in zebra blood collected from the various parks in South Africa as determined with real-time PCR. Affected park = where sarcoid has been observed; Unaffected park = where no sarcoid has been observed before; CM = Cape mountain zebra.

2.4 DISCUSSION

The South African Cape mountain zebras are descendants of 11 individual animals which originated from three populations at the Mountain Zebra National Park, Eastern Cape Province, the Kammanassie Nature Reserve, Western Cape Province and Gamka Mountain Nature Reserve, Western Cape Province (Bigalke 1952). These populations had been confined to fenced areas for many generations and the possibility exists that they are considerably inbred (Marais *et al*. 2007). Sarcoid lesions were first reported in Cape mountain zebras from the Gariep Dam Nature Reserve in June 1995 (Nel *et al*. 2006) and in 1998 in the Bontebok National Park (Lange 2004). In 2002, the incidence of sarcoid was reported to be 24.7% in the Gariep Dam Nature Reserve and 53% in the Bontebok National Park (Marais *et al*. 2007; Nel *et al*. 2006). A study by Sasidharan (2005), has shown specific heterozygote deficiency within these two populations of Cape Mountain zebras. It was also evident that the genetic diversity of these small and isolated populations of Cape Mountain zebras is reduced which therefore possibly accounts for the high prevalence of sarcoid tumours (Sashidharan, 2005).

Sarcoid is a common dermatological skin lesion that occurs in the skin of horses, donkeys and mules, in which it is generally associated with BPV types 1 and 2 (Angelos *et al*. 1991; Bloch *et al*. 1994; Reid *et al*. 1994; Nasir *et al*. 1997, Nasir & Reid, 1999; Chambers *et al*. 2003b). This study was undertaken to investigate if BPV is also present in sarcoid in the free-roaming zebra populations of South Africa by using conventional PCR, targeting the E5 ORF of BPV, RFLP analysis as well as real-time PCR to detect and distinguish between BPV-1 and -2.

The conventional PCR, as originally described by Teifke and Weiss (1991) was able to detect BPV DNA in the sarcoid tumours collected from the Cape mountain zebras from the Gariep Dam Nature Reserve, Bontebok National Park and Mountain Zebra National Park. Subsequent RFLP analysis on these amplified products revealed that, of the 12 sarcoid tumours investigated, 58% were derived from BPV-1 infection, 33% from BPV-2 and 9% (1 tumour) had both BPV-1 and -2 DNA present. No amplification product could be detected in the healthy skin or blood of any of these animals. To ensure the integrity of the DNA and to eliminate possible false negative results, control primers (Szalai *et al*. 1993; Fraser & Bailey 1996) were used to successfully amplify zebra class II major histocompatibility loci DRB and DQB (data not shown). This indicated that the BPV DNA concentration in the zebra blood and healthy skin was too low to be detected by conventional PCR.

In order to obtain a more sensitive PCR detection assay and to avoid RFLP analysis, a hybridization probe real–time PCR assay was developed to rapidly detect and differentiate between of BPV-1 and -2 DNA. Two separate hybridization probe sets were designed and used in a multiplex format for the specific detection of BPV-1 and -2 DNA. Although the test has proved to be extremely sensitive and able to detect as little as five genome copies, the BPV-1 and -2 hybridization probe sets always produced an amount of cross-reactivity which was observed as double peaks during melting curve analysis. PCR optimization could not eliminate this phenomenon. The melting temperature (T_m) of double stranded DNA molecules depend on the nucleotide sequence, length and the GC content. Hybridization probe melting temperature is dependent on (i) external factors such as salt concentration and pH and (ii) intrinsic factors such as concentration duplex length, GC content, and nearest neighbour interactions (Hermann *et al*. 2000; Lyon 2005). These differences can be detected by monitoring the fluorescence while increasing the temperature. The melting curve is visualized by a loss of fluorescence as the probes dissociate from the DNA template (Lyon, 2005). Nucleotide mismatches between the oligonucleotide probe and the DNA target result in a weaker thermodynamic bond between the amplified DNA and the probe and will cause the probe to dissociate at a significantly lower temperature (Lyon 2005). In this study, both hybridization probe sets which were designed for the specific detection of BPV-1 and -2 DNA yielded double melting peaks. In this case it was not due to mismatched bases in the probe region or due to possible mixed infection. This was evident by cloning and sequencing results which revealed no sequence variations in the targeted region. In addition, the same melting profiles were observed when plasmids containing cloned BPV-1 or -2 E5 ORF genes were subjected to the real-time PCR assay. The most probable explanation for this phenomenon is a too high input DNA concentration. When diluted DNA samples were subjected to the assay, a single peak could be observed in lower concentrated samples. Other possible reasons for double peaks are (i) because of the probe chemistry, the probes may melt off the target at an uneven rate resulting in double peaks (Sibeko *et al*. 2008) or (ii) the back-folding of the amplicon on itself downstream of the sensor probe which will result in competition with the probe, creating a lowered melting peak (Simpson *et al*. 2007).

The real-time PCR results of the 12 sarcoid tumour samples investigated were in concordance with the results obtained with the RFLP analysis. Furthermore, we were able to demonstrate the presence of BPV DNA (either as single or mixed infections) in the skin and/or blood of sarcoid-affected and healthy zebras in sarcoid-affected parks. Interesting is the presence of BPV DNA in the blood collected from zebras from parks where no sarcoid has been observed before. No mixed infections were observed in these specimens. An overall observation was that most infections (in both affected and unaffected parks) were due to BPV-1.

Several authors have demonstrated the presence of BPV DNA in whole blood of infected cattle (Campo *et al*. 1994; Campo 1998; De Freitas *et al*. 2003; Wosiacki *et al*. 2005) which could suggest vertical virus transmission via the blood stream (Brandt *et al*. 2008). Up to recently, BPV DNA could not be demonstrated in blood of BPV-infected equids (Nasir *et al*. 1997; Bogaert *et al*. 2008; Brandt *et al*. 2008). Recently, Brandt and colleagues (Brandt *et al*. 2008) demonstrated BPV DNA in peripheral blood mononuclear cells (PBMCs) obtained from sarcoid-affected horses. Their results support the hypothesis that PBMCs may serve as host cells for BPV DNA, contributing to virus latency (Brandt *et al*. 2008). In this study, we demonstrated the presence of BPV DNA in blood of sarcoid-affected and -unaffected zebra. This can be attributed to the highly sensitive nature of the real-time PCR assay used. Results showed that the concentration of BPV DNA in blood samples was much lower than that in the sarcoid tumours, as evident by the respective threshold values (Ct) of the amplification curves. The average Ct value obtained from sarcoid tissue was 21.65, which corresponds to ~2,25x10^6 genome copies. In contrast, the average Ct value of the positive skin samples was 35.2 (~25 genome copies) and that of blood from sarcoid-affected zebra was 37.8 (~1.5 genome copies). The Ct value of blood from sarcoid-unaffected zebra (from parks where no sarcoid has been observed before) was even lower with an average value of 39.07.

The presence of BPV DNA in healthy skin confirms the results obtained by Carr *et al*. (2001a) who demonstrated BPV DNA in 98% of equine sarcoid tissue and in 63% of normal skin from sarcoid positive animals which differ from earlier results where normal skin and other tissues from sarcoid-affected horses were consistently negative when tested for BPV DNA (Carr *et al*. 2001b; Martens *et al*. 2001). Bovine papillomavirus DNA was present on the sarcoid surface as well as normal skin of affected horses but also on 44% of non-affected horses living in contact with the affected horses (Bogaert *et al*. 2005). These authors did not test for BPV DNA in horses not in contact with sarcoid.

Recently, Bogaert and co-workers (Bogaert *et al*. 2008) also demonstrated BPV DNA in the normal skin of 73% horses with clinical sarcoid and in the normal skin of 73% horses in contact with cattle. The horses in contact with the clinical sarcoid group also demonstrated BPV DNA in normal skin (50%). In the control group, which had no contact with clinical sarcoid or cattle, 30% were positive for BPV DNA. The virus could not be detected in peripheral blood samples.

The immune response to equine sarcoid in the horse has not been fully investigated but O'Brien and Campo (2002) described several immune evasion mechanisms that may contribute to persistence and progression of papillomavirus-associated disease. If the BPV virus is present in both affected

and healthy animals, genetic predisposition may be why certain animals succumb to the disease (Ragland *et al*. 1966; Broström *et al*. 1988; Gerber 1989). Genetic predisposition was also shown in an association between certain MHC class II genes and the development of tumours induced in rabbits (Han *et al*. 1992) and in human cervical carcinoma associated with Human papillomavirus types 16, 18, 31 and 45 (Munoz *et al*. 2003). It is well described that conditions of immune suppression in humans lead to activation of latent infections or increased susceptibility to re-inoculation from active infections (De Villiers 1998). Immunodeficiency may also predispose animals to develop papilloma as seen in domestic cats (Sundberg *et al*. 2000) and various cat species kept in captivity in zoos (Sundberg *et al*. 1996). Animals held in captivity may be prone to immune suppression as was shown in manatees in Florida which developed multiple cutaneous papillomas (Bossart *et al*. 2002). As mentioned above sarcoid has also previously been described in two cases of captive zebra (*E. burchelli boehmi*) (Löhr *et al*. 2005).

The two parks in which the sarcoid occurred in this study are the smallest of all the parks in South Africa, being 3 486 ha and 6 000 ha, respectively. This small area may predispose the animals to immune suppression when conditions deteriorate with consecutive development of sarcoid.

The Bontebok National Park obtained a higher therapeutic rate on the treatment of equine sarcoids after using their autogenous vaccine than the Gariep Dam Nature Reserve which had several cases that did not respond to the vaccine (personal communication: Drs Pierre Nel and Dave Zimmerman). Using real-time PCR we were able to show that the Bontebok National Park's vaccine contained both BPV-1 and -2 whereas the Gariep Dam Nature Reserve's vaccine had only BPV-1 DNA present. We can only speculate that the zebras which did not respond to vaccination might have been infected with BPV-2 and that the vaccine did not cross protect between BPV-1 and -2.

In conclusion, we developed a rapid, sensitive and reliable real-time PCR to detect and distinguish between BPV-1 and -2 infections in zebras. We have shown that BPV-1 and -2 DNA (either single or mixed infections) are present in sarcoid tumours, healthy skin and blood of sarcoid-affected and healthy zebras from sarcoid-affected parks. This investigation also confirmed the high prevalence of sarcoids in Cape mountain zebras in the Gariep Dam Nature Reserve and Bontebok National Park. The most significant finding was, however, the presence of BPV-1 or -2 DNA in the blood of zebras from parks where no sarcoids have previously been observed.

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CHAPTER THREE:

PHYLOGENY OF PAPILLOMAVIRUSES OBTAINED IN SKIN LESIONS OF CAPE MOUNTAIN ZEBRA (*Equus zebra zebra,* Linnaeus, 1758)

ABSTRACT

After clinical, histological and molecular diagnosis of sarcoid in free roaming Cape mountain zebra (*Equus zebra zebra*), phylogenetic analysis of the BPV DNA in the E2E5 region demonstrated that sequences corresponded to variants of either BPV-1 or BPV-2. Sequences obtained from lesions in Equidae cluster together as sister taxa to the rest of BPV-1 sequences obtained from Bovidae. The phylogenetic position of BPV-1 and BPV-2 is well nested within a supertaxon containing papillomavirusses infecting Artiodactyla and Equidae. The evolutionary analysis estimate that the most recent common ancestor for all BPV-1 dates back to 1.4 million years (Mya) and in BPV-2 0.55 Mya. While the the last common ancestor for BPV-1 and BPV-2 dates back to 5.34 Mya.

Keywords: Papillomavirus, phylogeny, E2E5, Cape mountain zebra (*Equus zebra*), South Africa

3.1 INTRODUCTION

Papillomaviruses are classified in the family *Papillomaviridae* (Bernard, 2006) and cause disease in humans and numerous animal species by stimulating epithelial proliferation. Papillomaviruses are quantitatively the most important group of viruses associated with benign and malignant neoplasia in humans (Zur Hausen 1991). In animals papillomavirus association has been documented in various species: equids (Angelos *et al*. 1991; Bloch *et al*. 1994; Reid *et al*. 1994; Chambers *et al*. 2003; Löhr *et al*. 2005), felidae (Sundberg *et al*. 2000; Munday *et al*. 2008) camelids (Schulman *et al*. 2003); possums (Perrott *et al*. 2000); giraffes and impalas (Karstad & Kaminjolo 1978), and have been best studied in horses (Chambers *et al*. 2003) and cattle (Borzacchiello & Roperto 2008; Nasir & Campo, 2008). Animal papillomavirus have been studied as agents of disease in animals and as models of human papilloma infection (Campo 2002; Breitburd *et al*. 2006).

The papillomavirus genome is a single molecule of double-stranded, circular DNA and contains approximately 7 900 bp. Eight well defined open reading frames are encoded: L1 and L2 that encode capsid proteins and E1, E2, E4, E5, E6 and E7 that encode proteins involved in replication, transcription and transformation, and a non-coding, regulatory, long control region (LCR) (Bernard *et al*. 2006; Bravo & Alonso 2007). In humans, more than 100 different papillomavirus types, associated with both benign and malignant conditions, have been fully sequenced (De Villiers *et al*. 2004).

Papillomaviruses are thought to be highly species-specific. This hypothesis is, however, frequently rejected as papillomaviruses infecting the same host are not monophyletic, and many distantly related papillomaviruses infect the same host species (Bravo *et al*. 2010). The only known exception being naturally occurring cross-species infections of horses, donkeys and mules with BPV-1 and BPV-2 (Nasir *et al*. 2007) in which they are associated with sarcoids (Lancaster & Olson 1980; Nasir & Reid 1999; Campo 2002). Recently BPV was described in other examples of cross species infection when it was described in the water buffalo (*Bulbalus bulbalus*) (Silvestre *et al*. 2009) and in a European bison (*Bison bonasus*) (Literák *et al*. 2006). Kidney & Berrocal (2008) described sarcoids in tapirs (*Tapirus bairdii*) which histologically resembled equine sarcoids and BPV-1 was demonstrated. Munday *et al*. (2007) described a growth in a domestic cat (*Felis catus*) of which sequencing of the amplicon revealed 98% similarity to human papillomavirus type 9. In a study by Zaugg *et al*. (2005) papillomaviruses found in dogs (*Canis lupis familiaris*) were closely related to human papillomavirus type 5. Papillomaviruses are highly diverse and are likely occur in most mammals and birds (de Villiers *et al*. 2004). 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 have been historically assumed to have evolved alongside their hosts (Bernard 1994, Antonssun & McMillan 2006). Phylogenetic studies also suggest that they do not change host species, do not recombine, and have maintained their basic genomic organization for more than 100 million years (Bernard 2006). It has, however, been shown that phylogenetic trees of the viruses and their hosts are not congruent and that this hypothesis is questionable (Bravo *et al*. 2010).

The purpose of this investigation was to study the phylogenetic relationships of the papillomaviruses DNA obtained from tumours in the Cape mountain zebra (*Equus zebra zebra*), in South Africa.

3.2 MATERIALS AND METHODS

3.2.1 Study population and sample collection

Specimens of sarcoid tumours included in this study were obtained from Cape mountain zebras (*Equus zebra zebra*) from the Gariep Dam Nature Reserve (Free State Province) (n=9), Bontebok National Park (Western Cape Province) (n=2) and Mountain Zebra National Park (Eastern Cape Province) (n=1). Additionally, blood samples were collected from healthy, sarcoid-unaffected zebras (n=51) in these parks as well as from parks where sarcoids had never been observed.

The sarcoid tumour samples were collected by means of a biopsy punch. Each comprised a 10 mm x 3 mm x 3 mm piece of tissue which was stored in a glass tube at 4 °C until further analysis. Blood samples were collected in EDTA-buffered tubes by venipuncture of the jugular vein and were also stored at 4 °C.

Histopathologically confirmed equine sarcoid (n=1) and bovine papilloma (n=1) specimens were obtained from private veterinarians and were included in the study to serve as positive control material.

3.2.2 DNA extraction

DNA was extracted from 200 µl of blood or 25 mg of tissue (sarcoid) 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.

3.2.3 Conventional PCR amplification of a region of the E5 open reading frame

Conventional PCR was used for the amplification of 637 bp DNA fragment of a region of the E5 ORF of both BPV-1 and BPV-2 as described in Chapter 2.

3.2.3.1 Real-time PCR

The hybridization probe real-time PCR assay (as described in Chapter 2) developed for the detection and differentiation of BPV-1 and BPV-2 DNA in specimens was used to analyse the samples.

3.2.3.1.1 Primer and hybridization probe design

The conventional PCR primer set, the hybridization probes, PCR amplification mixture and reaction condition used were as described in Chapter 2. Specimens from histopathologically confirmed equine sarcoid from a horse (n=1) and a bovine papilloma (n=1) were included as positive controls.

3.2.3.2 Molecular cloning and sequencing

Conventional PCR was used for the amplification of 637 bp DNA fragment of a region of the E5 ORF of both BPV-1 and BPV-2. The primers (F_3610: 5'-GCT AAC CAG GTA AAG TGC TAT C-3'; R_4247: 5'-TGC TTG CAT GTC CTG TAC AGG T-3'), PCR amplification mixture and reaction conditions were as described in Chapter 2. The obtained amplicons were purified, cloned into the pGEM®-T vector (Promega pGEM–T Easy Vector System, Promega, Madison, USA), and transformed into competent JM 109 *E. coli* cells (Promega, Madison, USA) as described in Chapter 2. 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). Sequences were assembled and edited using the GAP4 program of the Staden package (version 1.6.0 for Windows) and aligned with published sequences of related genera using ClustalX (version 1.81 for Windows) (Staden *et al*. 2000).

3.2.3.3 Phylogenetic analysis

The phylogenetic position of BPV-1 and -2 and the relationships with its close relatives were reconstructed based on a concatenated alignment of the E1E2L1 genes, as described by Gottschling

et al. (2007) and Bravo *et al*. (2010). Briefly, the sequences were aligned at the amino acid level with MUSCLE (http://www.drive5.com/muscle/) (Edgar 2004), filtered for ambiguously aligned positions with GBLOCKS (http://molevol.cmima.csic.es/castresana/Gblocks.html) (Castresana 2000) visualized for manual correction and back-translated to the nucleotide level using PAL2NAL (http://www.bork.embl.de/pal2nal) (Suyama *et al*. 2006). Maximum Likelihood (ML) phylogenetic analysis was performed with RAxML v7.2.5 (http://wwwkramer.in.tum.de/exelixis/software.html) (Stamatakis 2006a) using the GTR+ model of evolution and the CAT approximation of rate heterogeneity (Stamatakis 2006b), which introduced nine partitions that corresponded to each of the codon positions of each of the three genes, and with 10 000 non-parametric bootstraps.

Fine relationships within this superclade, to which BPV belongs, were further reconstructed with the same procedure, while rerunning the GBLOCKS-filtering step using only members of the superclade and including RaPV1 as an outgroup. A dated Bayesian phylogenetic analysis was performed with BEAST v1.4.8 (http://beast.bio.ed.ac.uk) (Drummond & Rambaut 2007) with the GTR+ model of evolution, using an uncorrelated log normal relaxed clock, which introduced three partitions that corresponded to each of the codon positions, and unlinking parameters across codon positions. A uniform prior for the age of the most recent common ancestor of the superclade was fixed between 95.3 and 113 millions of years (Mya), based on the estimated dating for divergence time for the crown of Laurasiatheria (Bininda-Edmonds *et al*. 2007). Two independent chains of 50 million steps were calculated and analyzed with a burn-in of ten million steps and combined after a Bayes factor analysis on likelihood values for consistency (Suchard *et al*. 2001). The mutation rate was estimated to have an average of 0.0125 mutations per site per Mya (95% posterior density between 0.0110 and 0.0141). Values for average relative contributions to mutation rate were 0.49, 0.31 and 2.21, for each codon position respectively. The age for the most recent common ancestor for BPV-1 and BPV-2 was estimated to be 5.34 Mya (95% posterior density between 3.17 and 7.62), and this estimation was used in subsequent analyses.

A final phylogenetic analysis focusing on the genomic region analysed here and amplification was performed, including the C termini of the E2 gene, the E5a gene and the E5b gene (51, 56 and 69 parsimony informative positions, respectively). A dated Bayesian phylogenetic analysis was performed with BEAST v1.4.8 (http://beast.bio.ed.ac.uk) (Drummond & Rambaut 2007) with the GTR+ model of evolution, for both strict clock and uncorrelated log normal relaxed clock, introducing three partitions that corresponded to each of the codon positions, and unlinking parameters across codon positions. A normal prior for the age of the most recent common ancestor of BPV-1 and BPV-2 superclade was fixed with average 5.3 Mya and standard deviation 1.0 Mya.

There were no differences in the likelihood values for the trees obtained under strict clock and under relaxed clock assumptions, and the simpler, strict clock model was preferred. Mutation rate was estimated to have an average of 0.017 mutations per site per Mya (95% posterior density between 0.009 and 0.026). Values for average relative contributions to mutation rate were 0.996, 0.613 and 1.391, for each codon position respectively. Evolutionary relationships of the papillomaviruses were run and the hierachial taxonomonic relationships were constructed at Genomics and Health, Centre for Public Health Research, Valencia, Spain.

3.3 RESULTS

3.3.1 Real-time PCR

The real-time PCR results are shown in **Table 2** (Chapter 2). DNA obtained from all sarcoid tissue specimens tested positive for the presence of BPV DNA. For the blood samples collected from zebra from sarcoid-affected parks, 52% was positive for the presence of BPV DNA. Surprisingly, of the blood samples collected from zebras from sarcoid-unaffected parks, 41% were positive.

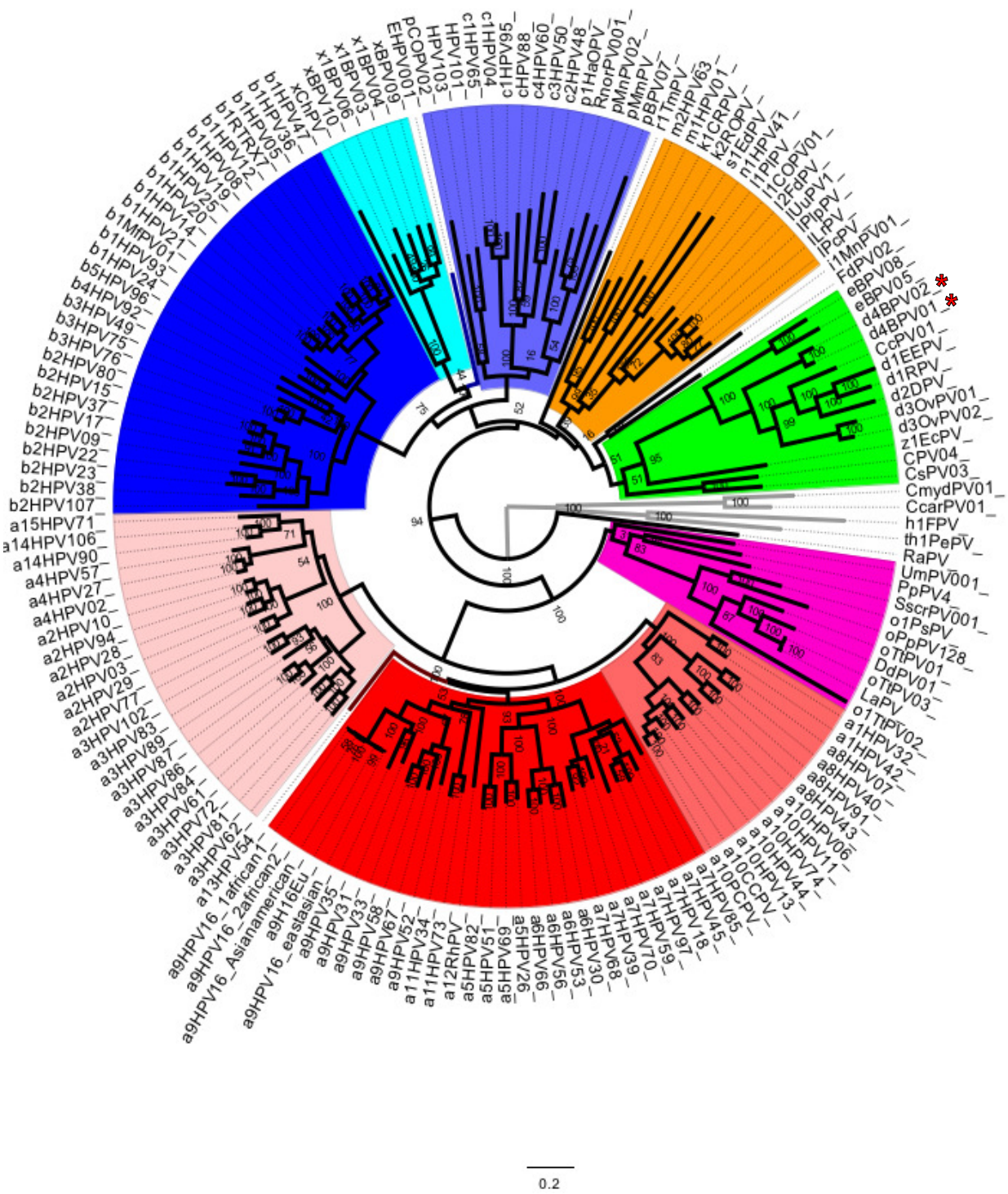
3.3.2 Sequencing results

A 637 bp amplicon of a region of the BPV E5 ORF could be amplified from all the zebra sarcoid tumour samples (n=12) using conventional PCR. Unfortunately, no amplification product could be obtained from blood samples using conventional PCR. The PCR amplicons obtained from the sarcoid tissue were cloned and sequenced. The obtained sequences were deposited in GenBank (accession numbers HQ541333-HQ541354). The number of base differences per sequence from analysis between sequences were conducted in MEGA4 (Tamura *et al*. 2007). All results are based on the pairwise analysis of 11 sequences. There were a total of 456 positions in the final dataset.

3.3.3 Phylogenetic reconstruction

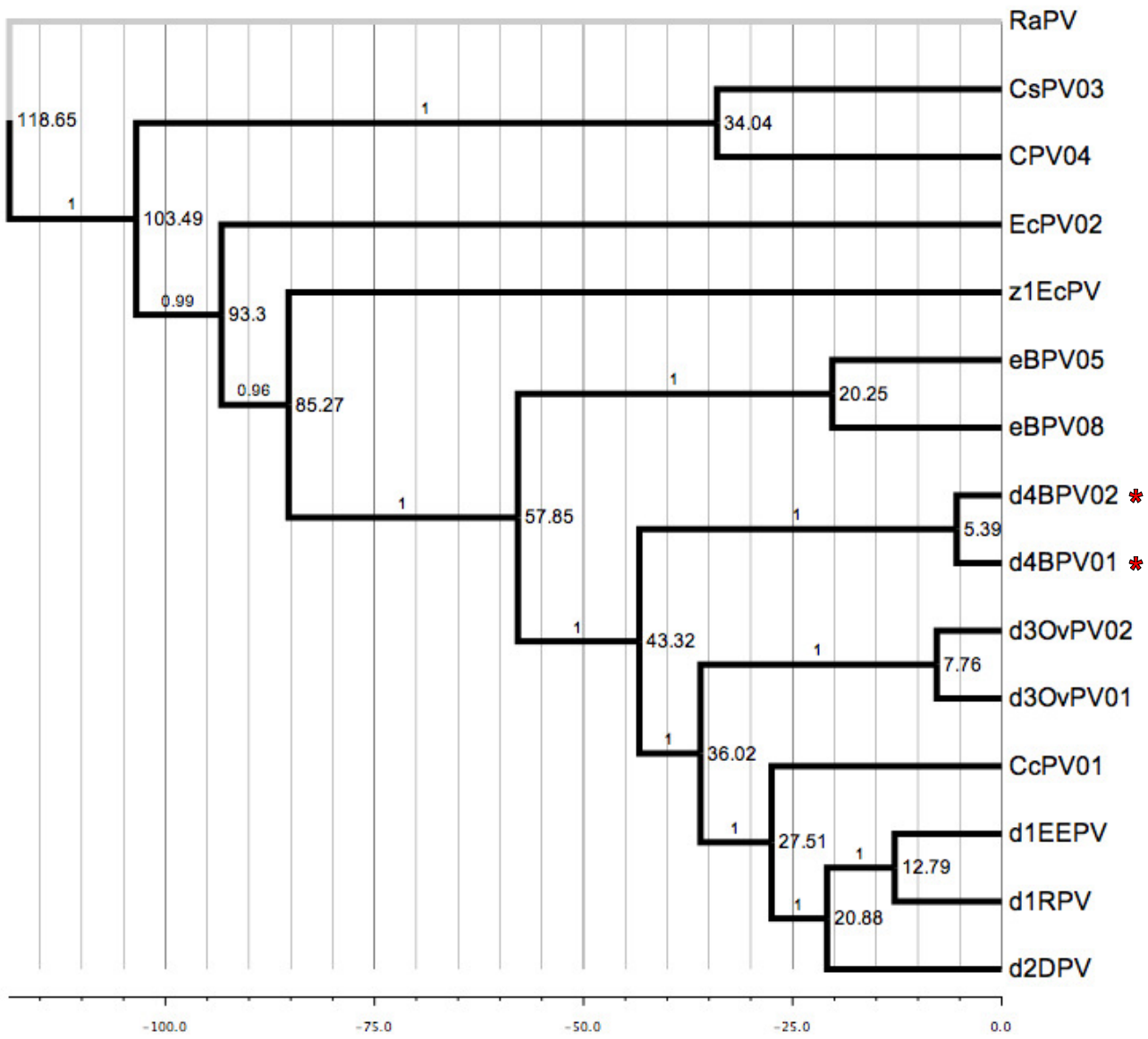
In a broad selection of papillomaviruses compiled in a phylogenetic tree (**Figure 10**) the phylogenetic position of BPV-1 and BPV-2 is well nested within the D superfamily (in green) (Gottschling *et al*. 2007; Bravo & Alonso 2007) which contains different papillomaviruses infecting the dog (CsPV3 and CPV4), horse (EcPV1 and EcPV4), cow (BPV-1, BPV-2, BPV-5 and BPV-8), sheep (OvPV1 and OvPV2), European elk (EEPV), roe deer (CcPV), deer (DPV) and white-tailed deer (RPV). This tree was constructed using the concatenated E1-E2-L1 genes, aligned at the amino acid level with back-translated into nucleotides, and calculated with RAxML. Support after

1 000 bootstrap replicatesis given on each nodes. Color codes highlight the four papillomavirus supertaxa. The tree was rooted with sequences from birds and turtles in grey. The scale bar is measured in substitutions per site. Bovine papillomavirus-1 and BPV-2 belong to the supertaxon encompassing delta, epsilon and zeta papillomavirusses, infecting different species within Laurasiatheria. This is consistent with previous descriptions; BPV-1 belongs unambiguously to a large clade encompassing members of delta, epsilon, zeta, dyoeta and chi-papillomaviruses (Chan *et al*. 1995; Bravo & Alonso 2007; Gottschling *et al*. 2007; Bravo *et al*. 2010; Vanderstraeten *et al*. 2010). The zebra sequences used in this study showed the highest similarity with BPV-1 and BPV-2 and their positions are indicated with an asterix in **Figure 10**.



*Indicates the position of the zebra samples from this study

Figure 10 Best known maximum likelihood phylogenetic tree for papillomaviruses.



*Indicates the position of the zebra samples from this study

Figure 11 Bayesian phylogenetic reconstruction of the subfamily D (green section in the previous figure) for sequences grouping together with BPV-1 and BPV-2 in the global papillomavirus tree.

A Bayesian phylogenetic reconstruction of subfamily D papillomavirus tree demonstrates the divergence time estimates for this subfamily D (the green section in the previous figure) in **Figure 11**. This tree has been rooted with the bat papillomavirus (RaPV) sequence (in grey) as an outgroup showing divergence time of 95.3-113 million years based on estimated dating for the divergence time for the crown of the superorder Laurasiatheria (Bininda-Edmonds *et al*. 2007). The position of the zebra sequences in this study, with BPV-1 and -2, is indicated by an asterix. An explanatory value for the abbreviations used in the tree is given in **Table 3**. The age for the most recent common ancestor for BPV-1 and BPV-2 was estimated to be 5.34 Mya (95% posterior density between 3.17 and 7.62).

Table 3 Abreviation, genus name of papillomavirus and host species used in **Figure 11**.

Abreviation	Genus papillomavirus	Host species	Family	Order
RaPV	Alpha papillomavirus	Bat papillomavirus		
CsPV03	Omikron papillomavirus	Dog papillomavirus	Canidae	Carnivora
CPV04	Alpha papillomavirus	Dog papillomavirus		
EcPV02	Alpha papillomavirus	Horse papillomavirus	Equidae	Perrisodactyla
Z1EcPV	Zeta papillomavirus	Horse papillomavirus		
eBPV05: eBPV08	Epsilon papillomavirus	Cow papillomavirus	Bovidae	Cetartiodactyla
d4BPV02: d4BPV01	Delta papillomavirus	Cow papillomavirus		
d3OvPV02: d3BPV01	Delta papillomavirus	Sheep papillomavirus	Ovidae	
CcPV01	Zeta papillomavirus	Roe deer papillomavirus	Cervidae	
d1EEPV	Delta papillomavirus	European elk papillomavirus		
d1RPV	Delta papillomavirus	White-tailed deer papillomavirus		
d2DPV	Delta papillomavirus	Deer papillomavirus		

Reference: Genomics and Health, Centre for Public Health Research, Valencia, Spain.

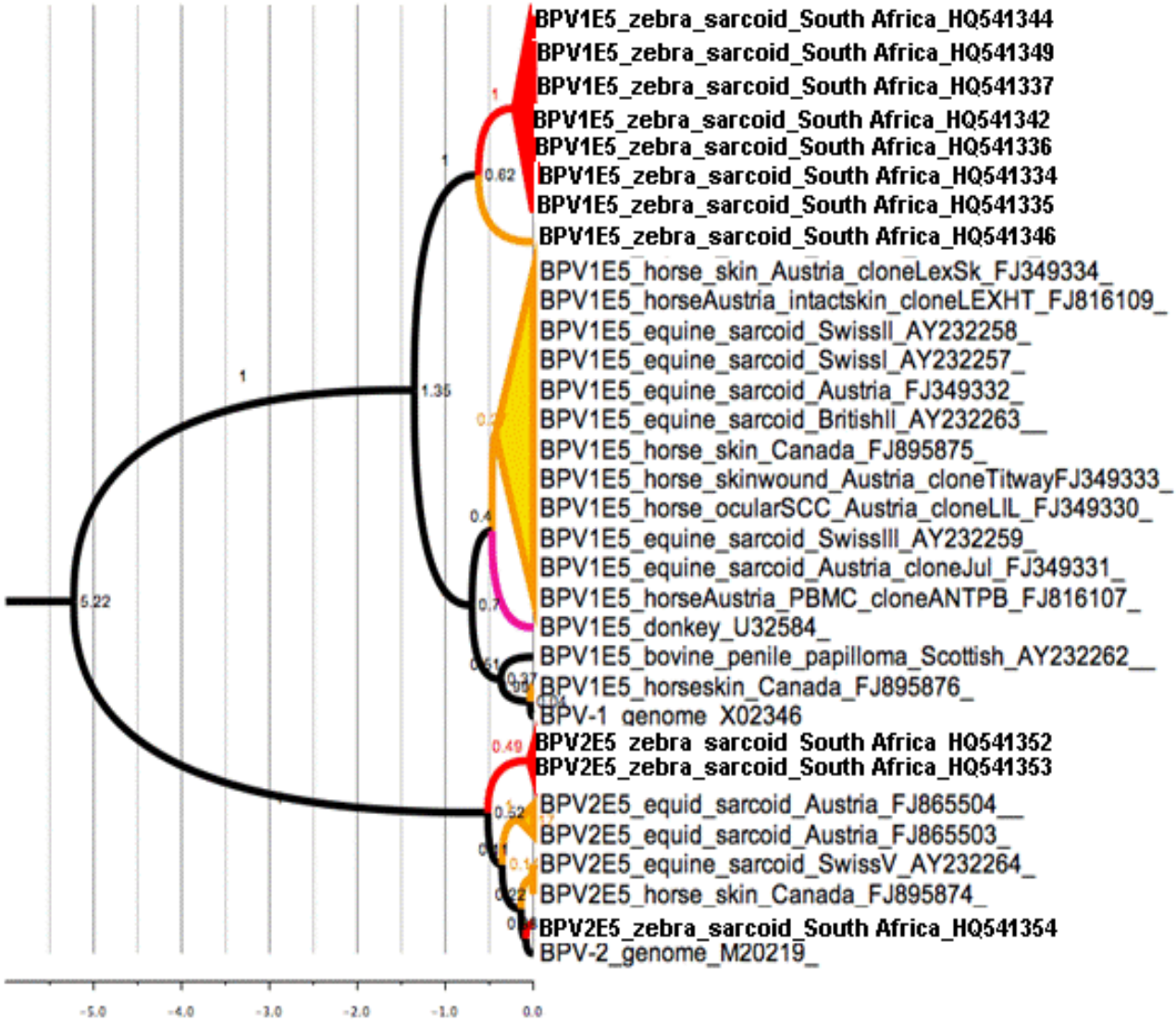


Figure 12 A Bayesian tree for BPV-1 and BPV-2 obtained from different animal species. Values on branches account for Bayesian posterior probability. Values on nodes account for node age median value. Colour coded is according to the host species (horse, orange; donkey, magenta; zebra, red; cow, black).

The different sequences of BPV-1 and BPV-2 are demonstrated in **Figure 12**, clustered according to the host species. This tree is rooted and timed according to the position in the supertaxon tree. The zebra sequences are demonstrated in red; horse in orange; donkey in magenta and cattle in black. The zebra sequences form sister clades to both BPV-1 (accession numbers HQ541334, HQ541335, HQ541336, HQ541337, HQ541342, HQ541344, HQ541346, HQ541349,) and BPV-2 (accession numbers HQ541352, HQ541353) and another clade, to BPV-2 (accession number HQ541354). The age for the most recent common ancestor for BPV-1 variants was estimated to be 1.40 Mya (95% posterior density between 0.64 and 2.26), while for BPV-2 variants were estimated to be 0.55 Mya (95% posterior density between 0.17 and 0.98).

Table 4 Estimates of evolutionary divergence between the BPV-1 and BPV-2 sequences obtained from zebra. The number of base differences per sequence from analysis between sequences were conducted in MEGA4 (Tamura *et al*. 2007). Codon positions included were $1^{st} + 2^{nd} + 3^{rd}$ + Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 456 positions in the final dataset.

	1	2	3	4	5	6	7	8	9	10	11	12
1. BPV-1genome X02346												
2. HQ541336	17											
3. HQ541337	17	2										
4. HQ541349	15	2	2									
5. HQ541344	16	1	1	1								
6. HQ541342	16	1	1	1	0							
7. HQ541346	16	1	1	1	0	0						
8. HQ541335	17	2	2	2	1	1	1					
9. HQ541334	17	2	2	2	1	1	1	2				
10. BPV-2 genome M20219	57	66	66	66	65	65	65	66	66			
11. HQ541352	63	72	72	72	71	71	71	72	72	6		
12. HQ541353	62	71	71	71	70	70	70	71	71	5	1	
13. HQ541354	59	68	68	68	67	67	67	68	68	2	8	7

Estimated evolutionary divergences between the BPV-1 and -2 sequences obtained from zebra sarcoid were compared with published sequences by determining the number of base differences per sequence (**Table 4**). Some sequences were identical, whereas others differed from each other by between 1-72 bp. The BPV-1 sequences HQ541342, HQ541344 and HQ541346 were identical. The rest of the BPV-1 sequences (HQ541334, HQ541335, HQ541336, HQ541337, HQ541349, differed from the published BPV-1 sequence by 1-17 nucleotides. The obtained BPV-2 sequences (HQ541352, HQ541353, HQ541354) differed from the published BPV-2 sequences by only 2-6 nucleotides.

3.4 DISCUSSION

During the last several years the importance of knowledge about the evolution of pathogens such as papillomaviruses has been stressed (Bernard 2005; Bravo *et al*. 2010), as information about their phylogeny can contribute to their classification. If the evolutionary history of the papillomaviruses is understood, and how ancestral properties have been influenced, the clinical picture can be evaluated with respect to benign or malignant (van Ranst *et al*. 1992; Bravo & Alonso 2007).

Although most authors based phylogenetic studies on the BPV E1, E2 and L1 genes, E5 was used in this study. E5 is expressed in over 75% of sarcoid lesions suggesting that its expression is maintained throughout the disease (Nasir & Reid 1999; Carr *et al.* 2001a,b; Chambers *et al.* 2003).

Papillomavirus gene sequences retrieved from zebra sarcoid tumours can be identified as variants of either BPV-1 or BPV-2. Most BPV-1 sequences gained from non-bovine hosts cluster together and define sister taxa to canonical bovine associated BPV-1. The presence of BPV-1 sequences in zebra sarcoids highlights again the importance of this virus in the aetiology and pathogenesis of sarcoid in the Equidae family (Yuan *et al*. 2007).

Bovine papillomavirus -1 and -2 belonged unequivocally to the delta-epsilon-zeta papillomavirus superclade (**Figure 10**). Within this superclade, papillomavirus infecting Canidae (CsPV03 and CPV04) and infecting Cetartiodactyla (eBPV05, eBPV04, d4BPV02, d4BPV01, d3OvPV02, d3OvPV01, CcPV01, d1EEPV, d1RPV, d2DPV) were respectively, monophyletic i.e. they share a recent common ancestor (**Figure 11**). However, papillomavirus that infect horse and papillomavirus that infect cow are not monophyletic respectively. Strict host specificity of papillomaviruses prevents close contact between viruses but a variety of phylogenetically different hosts can be infected by BPV-1 and BPV-2, which display a wider host range, infecting close species such as the waterbuffalo (Silvestre *et al.* (2009), but also more distant species such as the horse (Bloch *et al.* 1994), donkey (Reid *et al.* 1994), Florida manatees (*Tricechus manatus latirostis*) (Bossart *et al.* 2002), tapir (Kidney & Berrocal 2008) and the zebra.

In **Figure 12** the sequences obtained from the zebras clustered together (red) and the other BPV-1 sequences (yellow) were all obtained from horse sarcoid or skin from all over the world. A different branch with BPV-1 was obtained from a donkey and another branch contained BPV-1 from bovine and horseskin. They could all relate to BPV-1 genome accession number X02346. In BPV-2 the sequences obtained from the zebras clustered in two branches (red) and again the other

BPV-2 sequences were obtained from horse skin or sarcoid samples. They could all relate to BPV-2 genome accession number M020219. The small differences in nucleotides in the zebra sequences, as seen in **Table 4**, play a role in this separation of zebra sequences.

The sequences analysed in this study were obtained from free-ranging zebra. The sarcoids originated from two game parks which rules human contact out as far as their playing a possible role in the transmission of the neoplasm. This is in contrast to the situation in horses and donkeys which are handled by humans or possibly share facilities with cattle or sheep in the normal daily farming activities. These zebras were, however, brought into the areas which had been previously inhabited by farm animals when the parks concerned were proclaimed in 1931 and 1972 respectively. Other game species share the grazing in the parks. Evolutionary incongruence is seen in BPV, these viruses infecting the same host (*Bos taurus*) are found in other species as well. For BPV-1, the last common ancestor was 1.35 Mya, and for BPV-2, 0.55 Mya, in this host switch, there was no human intervention as, for instance, could be caused by sharing the same facilities with horses and cattle. The other explanation could be that the common ancestor to BPV-1 and BPV-2, (5.34 Mya) already had the ability to infect other hosts species but was unable to gain effective access to them.

More information might have been obtained using the more variable upstream regulatory region (URR) that harbours transcription factor-binding sites and controls gene expression. It consists of a cluster of genes involved in the initial destabilization of the host cell (E4,5,6,7) and genome replication (E1,2) and a cluster of late genes that encode the capsid proteins where more BPV variants have been described. The E5, besides being detectable in a larger percentage of samples, can however, also be used in the phylogenic calculations (personal communication: Bravo).

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CHAPTER FOUR:

DEVELOPMENT OF A TYPING SYSTEM TO DETERMINE MHC HAPLOTYPES IN THE ZEBRA

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ABSTRACT

The outbreak of sarcoid in the Cape mountain zebra herds in South Africa, as the herds were becoming more inbred, raised the possibility of genetic influence. Since susceptibility to sarcoid has been described in different equine breeds all over the world in association with MHC haplotypes these herds were investigated for MHC types. Papillomavirus-induced tumours possessing associations with MHC class II haplotypes have also been encountered in other species. To establish an appropriate typing test, sufficiently simple to test a large amount of zebras, single strand conformational polymorphism (SSCP) of MHC class I and class II genes of zebras was investigated. Primers designed to amplify MHC class I and II genes on DNA samples of horses have been used for the amplification of MHC class I and II genes on DNA samples of *Equus zebra hartmannae*. The results demonstrated that class I genes were highly complex, class II DRA were not polymorphic among zebras and the primers for DQA failed to amplify products in the zebra. DRB and DQB could however be used to score SSCP gels with and without sarcoid.

Keywords: MHC, sarcoid, Cape mountain zebra, SSCP, class II genes DRB and DQB.

4.1 INTRODUCTION

Since 1937, after facing near extinction Cape mountain zebra herds are protected in a few game parks. One fundamental strategy for conservation of a species is to maintain the genetic diversity in a population so that the risk of the population going extinct is minimized, as loss of genetic diversity leads to decreased resistance to disease. The South African Cape mountain zebras are descendants of a nucleus of 11 individuals (Bigalke 1952) and the possibility exists that they become inbred with resultant loss of genetic diversity making them more vulnerable. The major histocompatibility complex (MHC) class I and class II genes play a key role in the initiation of the immune reponse (Klein 1986) and exhibit a high level of genetic polymorphism.

In 1995, equine sarcoid-like lesions appeared in 24.7% of Cape mountain zebras from the Gariep Dam Nature Reserve (Free State Province) (Nel *et al*. 2006) and in 1998 in the Bontebok National Park (Western Cape Province) in 53% of animals (Lange 2004). An isolated case of a zebra that was euthanized due to the severity of sarcoid lesions was reported in 2004 in the Mountain Zebra National Park (Eastern Cape Province) (personal communication: Dr Dave Zimmerman).

Sarcoid susceptibility in horses was found associated with MHC class I or class II antigens in family studies (Marti *et al*. 1996). In particular, horses with MHC class II haplotypes possessing the ELA-W13 serological specificity had an increased risk for sarcoid tumours (Lazary *et al*. 1985). Associations between class II MHC genes and papillomavirus-induced tumours have also been described in other species. In humans, papillomavirus-induced squamous cell carcinoma of the cervix is more common in women carrying the HLA-DQW3 class II MHC allele (Wank & Thomssen 1991). Apple *et al*. (1994) found a papillomavirus type-specific association between HLA DR-DQ haplotypes in this carcinoma of the cervix. Shope papillomavirus, which induces the development of tumours in the rabbit, are associated with class II MHC genes (Han *et al*. 1992).

The association of sarcoid tumors with MHC genes in horses was determined using serological typing for class I and class II MHC genes (Lazary *et al*. 1985; Broström *et al*. 1988). Equine MHC class I and class II genes are closely linked and exhibit linkage disequilibrium. Therefore, it was not possible to determine whether the association was for class I genes, class II genes or another of many genes linked to the MHC. Class I genes are a likely candidate for the association since they are implicated in recognition of virally infected cells (Klein, 1986). However, it is just not possible to determine which genes are implicated because of linkage disequilibrium and the close association of class I and class II genes in a limited number of haplotypes.

Serological studies of MHC in zebras were not possible since the existing reagents for horses did not recognize antigenic structures in zebras (Antczak and Bailey, personal communication). Therefore, MHC typing in zebras needed to be based on molecular approaches. The DNA sequences of genes are highly conserved among mammals. Molecular probes for human and mouse MHC class I and class II genes were effective to study horse MHC genes (Alexander *et al*. 1987). Likewise, PCR primers developed for human class II genes were effective to study horse class II genes (Szalai *et al*. 1993; Szalai, 1994; Szalai *et al*. 1994). Therefore, the premise for these studies was that the molecular tools, effective when used between humans and horses, would also be effective when used for zebras.

Several approaches were available at the beginning of this study, the most sensitive being the cloning and sequencing of MHC genes. However, tens of class I and class II genes were known to exist for horses (Alexander *et al*. 1987) and the cost and labor of approaching the work from this direction would be prohibitive. Fraser (1998) and Fraser and Bailey (1996; 1998) described a rapid and inexpensive approach to investigating genetic variation of equine MHC class II genes by using single strand conformational polymorphism (SSCP).

Single strand conformational polymorphism reproducibly detects genetic variation in DNA and has been used effectively to investigate MHC class II among horses (Fraser 1998). Therefore, the use of SSCP in an experimental approach to determine the extent of genetic variation for the MHC class II genes of zebras was also investigated.

These studies were designed to determine whether the approaches used to characterize variation for the equine MHC class II genes would also be efficient for zebras.

4.2 MATERIALS AND METHODS

4.2.1 Zebras and horse DNA samples for comparison

DNA samples (n=14) from Hartmann's mountain zebra (*Equus zebra hartmannae*) were received at Gluck Equine Research Center, Kentucky, from the Center for Reproduction of Endangered Species in San Diego California mainly for the development of a typing system of the zebra MHC. No family data was available on these zebra. For comparison, DNA isolated from peripheral blood cells from five unrelated domestic horses (*Equus caballi*) were used as controls. Comparisons of these two equid species were conducted at the Maxwell H. Gluck Equine Research Center at the University of Kentucky, USA.

4.2.2 Conventional PCR amplification of a second exon class II

Amplification of the class I and II (DRA, DRB, DQA and DQB) genes was performed using the primers as shown in **Table 5**. The amplified regions lie within the second exon of both genes and were used in genomic amplifications for SSCP typing. In class I, two zebras were used, in class II, DRA, two horses and nine zebra, in DRB two zebras and in DQB three zebras were used.

Table 5 Primers used for amplification of class I and II gene products.

Primer	Primer sequence	Bp	Reference
Classs I-2F	(5'-GCT CCC ACT CCA TGA G)	269 bp	
Class I-2R	(5'-GGC CTC GCT CTG GTT)		(Fraser 1998)
Class II DRA Be1	(5'-GGA TCC AGG CTG AGT TCT ATC TG)	229 bp	
Class II DRA-Be2	(5'-GGC TTA AGA GTG TTG TTG GAG CGC TT).		(Bailey 1994)
Class II DRB 2a	(5'-CTC TGC AGC ACA TTT CCT GGA G)	276 bp	
Class II DRB 2b	(5'-CGC CGC TGC ACC AGG AA)		(Fraser & Bailey 1996)
Class II DQA 2e	(5'-CTG AIC ACI TTG CCT CCT ATG)	246 bp	
Class II DQA 2f	(5'-TGG TAG CAG CAG IAG IGT TG)		(Fraser & Bailey 1998)
Class II DQB-GH28	(5'-CTC GGA TCC GCA TGT GCT ACT TCA CCA ACG)	210 bp	
Class II DQB-GH29	(5'-GAG CTG CAG GTA GTT GTG TCT GCA CAC)		(Szalai 1994)

The amplification mixture consisted of 2.0 µl DNA (~75ng), 0.2 µl *Taq* DNA polymerse (New England BioLabs inc) 1 µl 10X buffer with $MgCl_2$, 0.5 µl of each primer (Integrated DNA Technologies, Inc, Coralville, Iowa, USA), 1 µl (100 µM) dNTP's (Invitrogen, Carlsbad, California, USA) and nuclease free water to a total volume of 10 µl. The thermal profile for the amplification was as follows: initial denaturation was performed at 95 °C for 10 minutes. This was followed by 30 cycles, each consisting of 95 °C for 30 seconds, annealing at 56 °C for 30 seconds and extension was at and 72 °C for 30 seconds followed by a final extension at 72 °C for 10 minutes before cooling to 4 °C. The amplification was performed in an automated thermocycler (PerkinElmer, Foster City, CA).

4.2.3 Cloning and sequencing

The PCR amplicons obtained were purified and cloned into plasmid vectors using Invitrogen TA (Carlsbad, CA) cloning kit, and transformed into competent *Escherichia coli* cells (One Shot® INVαF' Invitrogen). Recombinant plasmid DNA was extracted and directly sequenced using the ABI BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) and analyzed on an ABI 3100 sequencer. Consensus sequences were compared to sequences obtained from GenBank using BLAST.

4.2.4 MHC Typing: Single strand conformational polymorphism analysis

Analysis was based on the SSCP method originally described by Orita *et al*. (1989). The SSCP gels consisted of a 10% w/v acrylamide:bis-acrylamide (37.5:1) (BioRad, USA) 0.5X TBE buffer (44.5mM boric acid (Merck, USA), 1mM Na_2-EDTA(Merck, USA), pH=8.4), 0.093% w/v ammonium persulphate (Merck, USA) and 0.08% v/v N,N,N',N'-tetramethylethylenediamine (TEMED) (Merck, USA). The samples consisted of PCR products from genomic DNA as well as cloned PCR products.

Samples (8 µl water + 3 µl loading dye { 95% formamide, 20mM Na_2-EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF}, + 2 µl PCR products) were denatured in a heating block at 90 °C for 10 minutes and put immediately on ice until loaded. The gels were electrophoresed in 0.5X TBE buffer on a Protean II xi cell gel apparatus (BioRad Laboratories Inc., Melville, NY) at 100 volts for 20 hours at 20 °C. The SSCP gel was visualized by staining for 10 minutes in 0.5X TBE with 1 ul of a 1 mg/ml stock solution of ethidium bromide, and photographed under UV trans-illumination. Gels were scored by counting the number of fragments visible on the gel (**Table 7**).

4.3 RESULTS

4.3.1 Sequencing

The equine-specific primers successfully amplified the 2nd exon of the MHC class I genes and class II genes DRA, DRB and DQB. These primers were not effective in amplifying the 2nd exon of DQA in the zebras.

4.3.1.1 Class I

The alignment of nucleotide sequences for the 2nd exon of the MHC class I genes for clones from two Hartmann's mountain zebras (*Ezh*) number 509 (four clones) and number 1568 (five clones) is shown in **Figure 13** as well as domestic horses {*Equus caballus (Ec)*} accession numbers DQ083420 and X79892.

Among four clones for zebra 509, there were clearly three different class I sequences. Among five clones for zebra 1586 there were four different sequences. A BLAST search showed that the highest similarity (~96%) occurred with MHC class I antigen *Ec* DQ083420 and (~94%) with *Ec* X79892, confirming that the products are MHC class I.

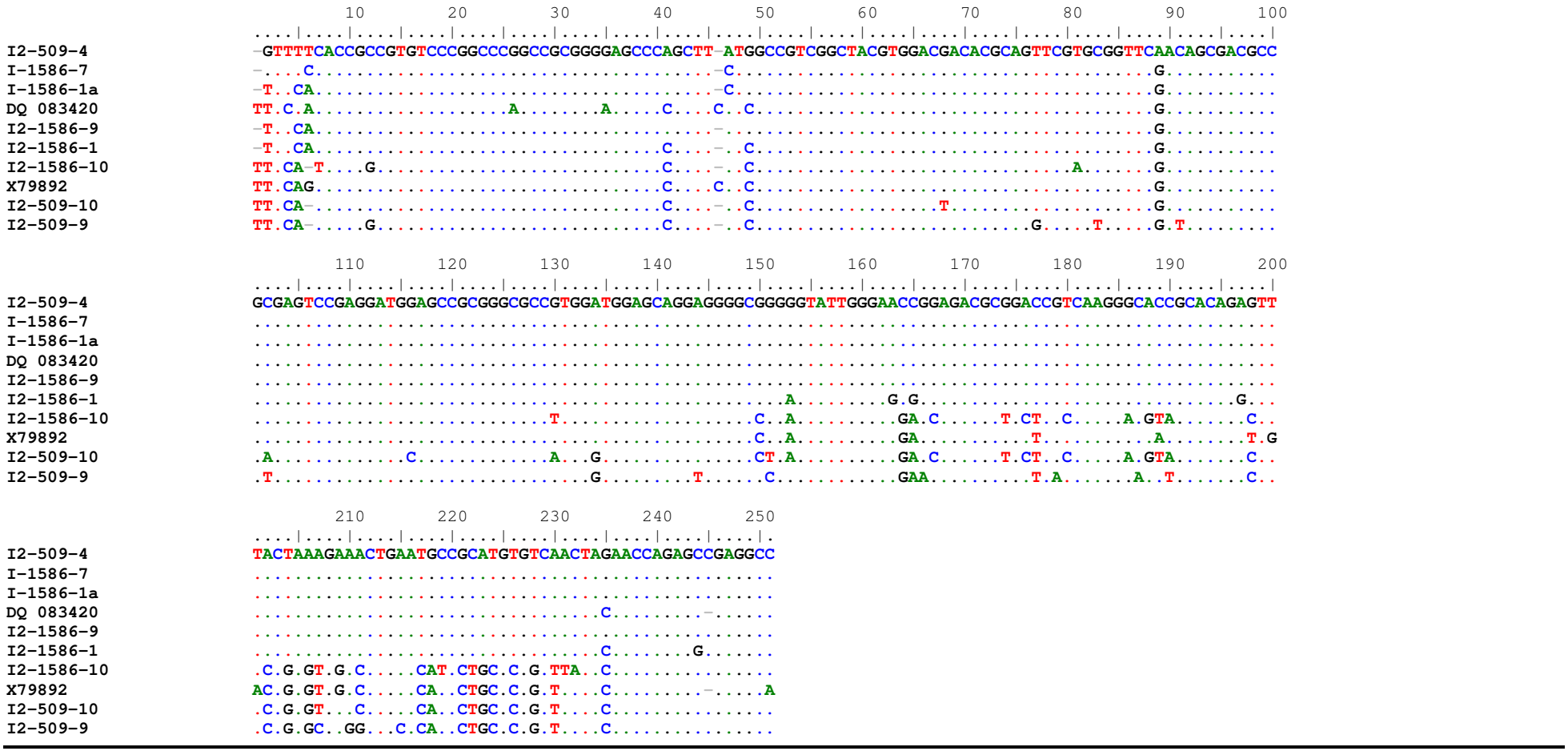

Figure 13 Alignment of nucleotide sequences for the 2nd exon of the MHC class I clones used for developing a typing system (designated "I2-animal number-clone number") from (*Ezh*) 509 and 1586. *Ec* accession numbers (DQ083420) and (X79892) are used as references.

4.3.1.2 Class II

4.3.1.2.1 DRA

All sequence data were consistent with a single locus for DRA. Clones (15) from three unrelated zebras (*Ezh* 132, 509, 510) were sequenced and no more than two different sequences were found. Subsequent sequencing of genomic DNA of five zebras (*Ezh* 10949, 6819, 3183, 454, 2040) in this study produced data consistent with a single locus. Sequences for clones or genomic sequences for Hartmann's zebras and control horses are demonstrated in **Figure 14** with reference sequences of *Ec* accession number M60100 and *Equus grevyi (Eg)* accession numbers EU930116, EU930124, EU930125. BLAST results revealed the highest similarity (~99%) with MHC class II DRA gene in *Eg* accession number EU930116 and (~98%) with *Ec* accession number M60100 and *Eg* accession number EU930125 and *Eg* accession number EU930124 confirming the products are MHC class II DRA. Alignment is raw data. Past sequence 210, the sequence overlaps with the site for primer Be2. The sequence reflects a discrepancy between the Be2 primer sequence and the genomic DNA sequence. Three alleles from 28 sequences are found.


10 20 30 40 50 60 70 80 90 100
DRA-509-3i
DRA-510-3i
DRA-510-2i
H2455
g132i
EU930125
EU930124
EU930116
M60100
DRA-10494
DRA-509-7
DRA-509-9
DRA-509-2
DRA-510-ii
DRA-132-a
DRA-132-b
DRA-6819
DRA-g509
DRA-g510
DRA-132-4
DRA-509-6
DRA-132-7
DRA-132-9
DRA-g132
DRA-132-ii
DRA-2040
DRA-g3183
DRA-3183
DRA-509-ii
DRA-454
DRA-132-4i
110 120 130 140 150 160 170 180 190 200

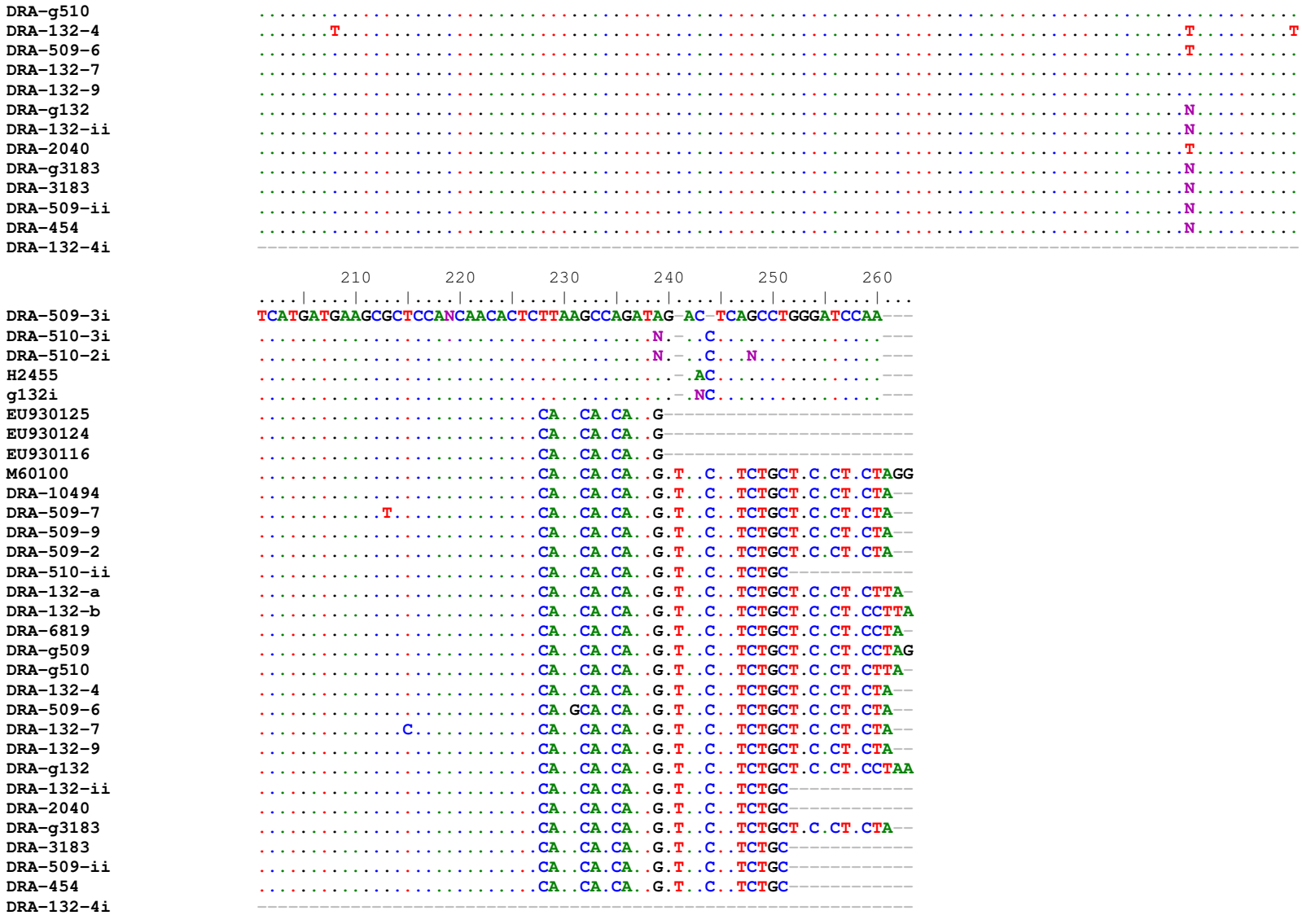

Figure 14 Alignment of nucleotide sequences for the 2nd exon of the MHC class II DRA clones (designated "DRA-animal number–clone number") or genomic sequence (designated "g" followed by animal number) from *Ezh* 132, 509, 510, 3183, 454, 10494, 2040, 6819, and horses H2455 and H2449. Reference sequences of *Ec* accession number (M60100) and *Eg* accession numbers (EU930116, EU930124, EU930125) are used.

In **Table 6** a summary of known DRA 2nd exon allele sequences plus the DRA alleles found among Hartmann's Zebras (prefix *Ezh*) and two horses, when typed by sequencing clones or sequencing genomic DNA are shown. The base present at different positions for each allele is shown with a listing of position number for sequence position obtained in this study followed by the position from Albright-Fraser *et al*. (1996) listed in parentheses. The Hartmann's zebras possessed the alleles previously identified by Fraser (1998) and Brown *et al*. (2004) as 0501, 0601 and JBZ185. The types for *Ezh* numbers 132, 509 and 510 were confirmed by sequencing clones of exon 2. The genotypes from the others appeared to be the result of a simple polymorphism at a single site (DNA base 188). The type for *Ezh* 7040 was deduced from genomic DNA as the simplest explanation for the genotype with the known DRA alleles; the type needs to be confirmed by cloning and sequencing.

Table 6 Summary of known DRA 2nd exon allele sequences plus the DRA alleles found among Hartmann's Zebras (prefix *Ezh*) and two horses when typed by either sequencing clones or sequencing genomic DNA.

ALLELE	**34(11)**	**83(93)**	**133(142)**	**138(144)**	**182(193)**	**188(199)**	**TYPE**
0101	G	C	G	G	C	C	0101
0201	G	C	G	A	C	C	0201
0301	G	C	A	G	G	C	0301
0401	G	C	A	G	C	C	0401
0501	G	T	G	G	C	C	0501
0601	A	C	G	G	C	C	0601
JBZ185	G	T	G	G	C	T	JBZ185
JBH11	G	C	G	G	C	T	JBH11
EQUID							
Ezh 10494	G	T	G	G	C	C	0501
Ezh 132	A/G	C/T	G	G	C	C/T	0601/JBZ185
Ezh 3183	G	T	G	G	C	C/T	0501/JBZ195
Ezh 454	G	T	G	G	C	C/T	0501/JBZ185
Ezh 509	G	T	G	G	C	C/T	0501/JBZ185
Ezh 510	G	T	G	G	C	C	0501
Ezh 6189	G	T	G	G	C	C	0501
Ezh 7040	A/G	C/T	G	G	C	T/C	?JBZ185/?601
Horse 2449	G	C	G/A	G/A	G/C	C	0201/0301
Horse 2455	G	C	G	A	C	C	0201

4.3.1.2.2 DRB

In the sequencing data of nine clones from zebra 510 and one clone from zebra 509 evidence for only two sequences was found. It is likely that zebra 509 has but a single DRB locus and shares an allele with zebra 510. The sequencing data are demonstrated in **Figure 15**. Two different *Ezh* consensus sequences (represented by clones 510-6 and 510-1) were compared to GenBank sequences using BLAST. No exact homology was found. However, the two sequences were most similar to DRB alleles found in Przewalski horses (*Equus ferus przewalski*) *Eqpr*-DRB*2 and *Eqpr*-DRB*5.

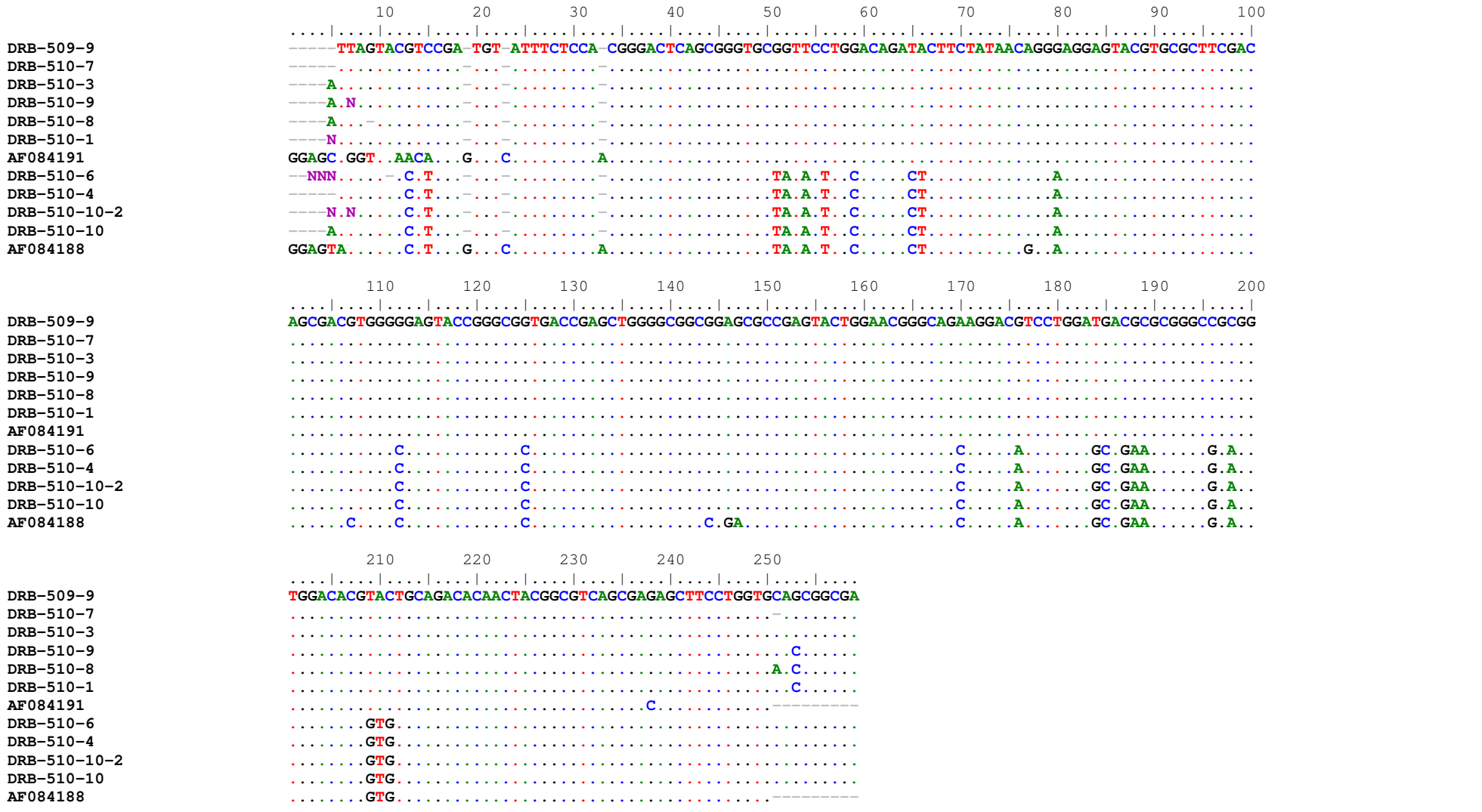

Figure 15 Alignment of nucleotide sequences for the 2nd exon of the MHC class II DRB clones (designated "DRB-animal number-clone number") from *Ezh* 510 and 509 as well as *Eqpr*AF 084188 and AF08491.

4.3.1.2.3 DQB

The data for DQB are consistent with the fact that two loci for DQB in these zebras exist. Zebra 132 had four sequences among seven clones tested, zebra 509 had four sequences among nine clones tested and zebra 510 had three sequences among six clones tested. The sequencing data are demonstrated in **Figure 16**. The data do not exclude the existence of more loci but are minimally compatible with two loci and complete heterozygosity for 132 and 509. As reference sequences *Equus asinus (Ea)* accession number (AF034125) and *Ec* accession numbers (L33910) are used with which BLAST results revealed highest similarity: *Ea* (~94%) and *Ec* (~93%) confirming that the products are MHC class II DQB.

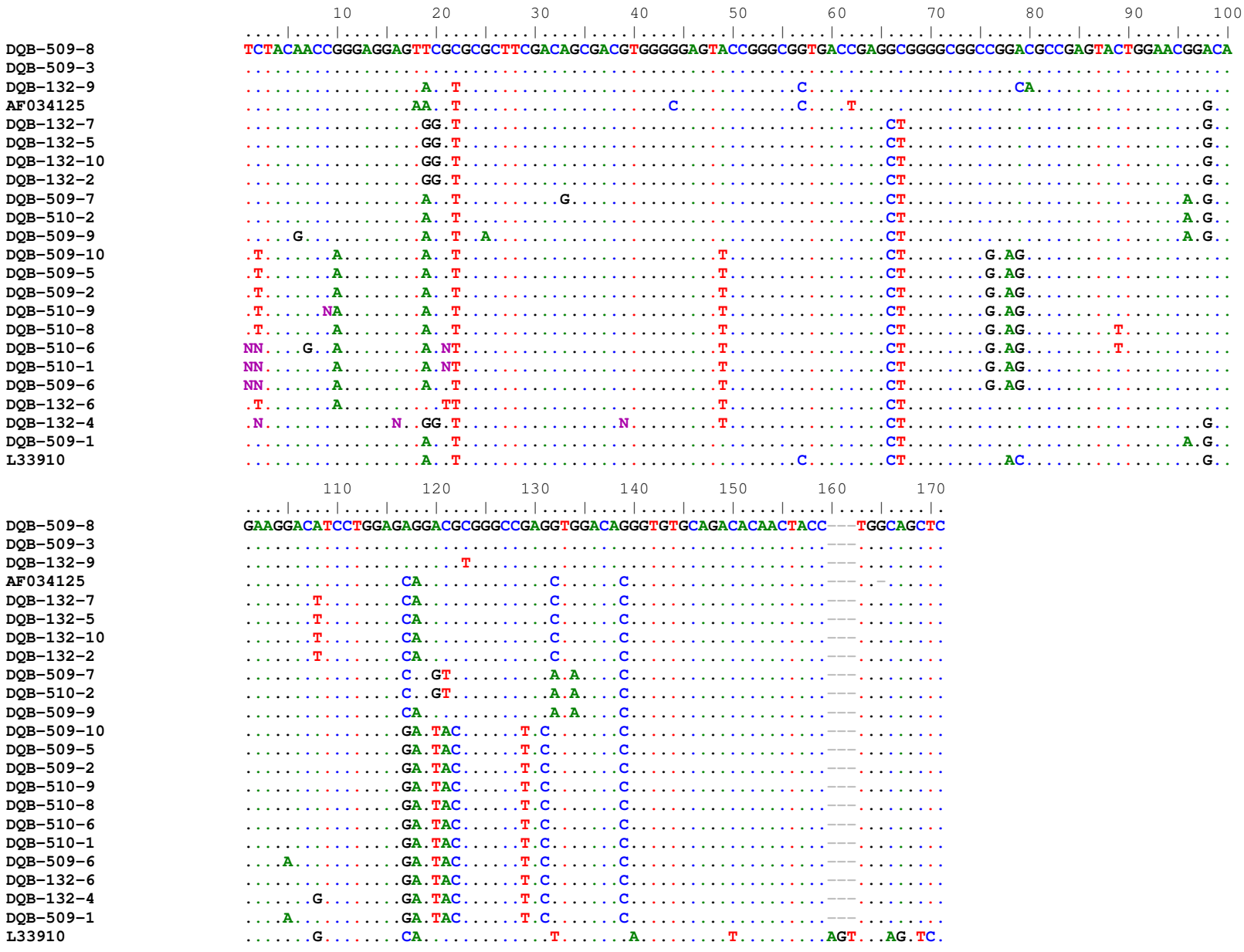

Figure 16 Alignment of nucleotide sequences for the 2nd exon of the MHC class II DQB clones (designated "DQB-animal number-clone number") from *Ezh* 132, 509 and 510. *Ea* accession number AF034125 and *Ec* accession number L33910 are used as references.

4.3.2 Single strand conformational polymorphism

4.3.2.1 DRB

Single strand conformational polymorphism testing was conducted with eight Hartmann's zebras and four horses. Polymorphic patterns were identified as shown in (**Figure 17**).

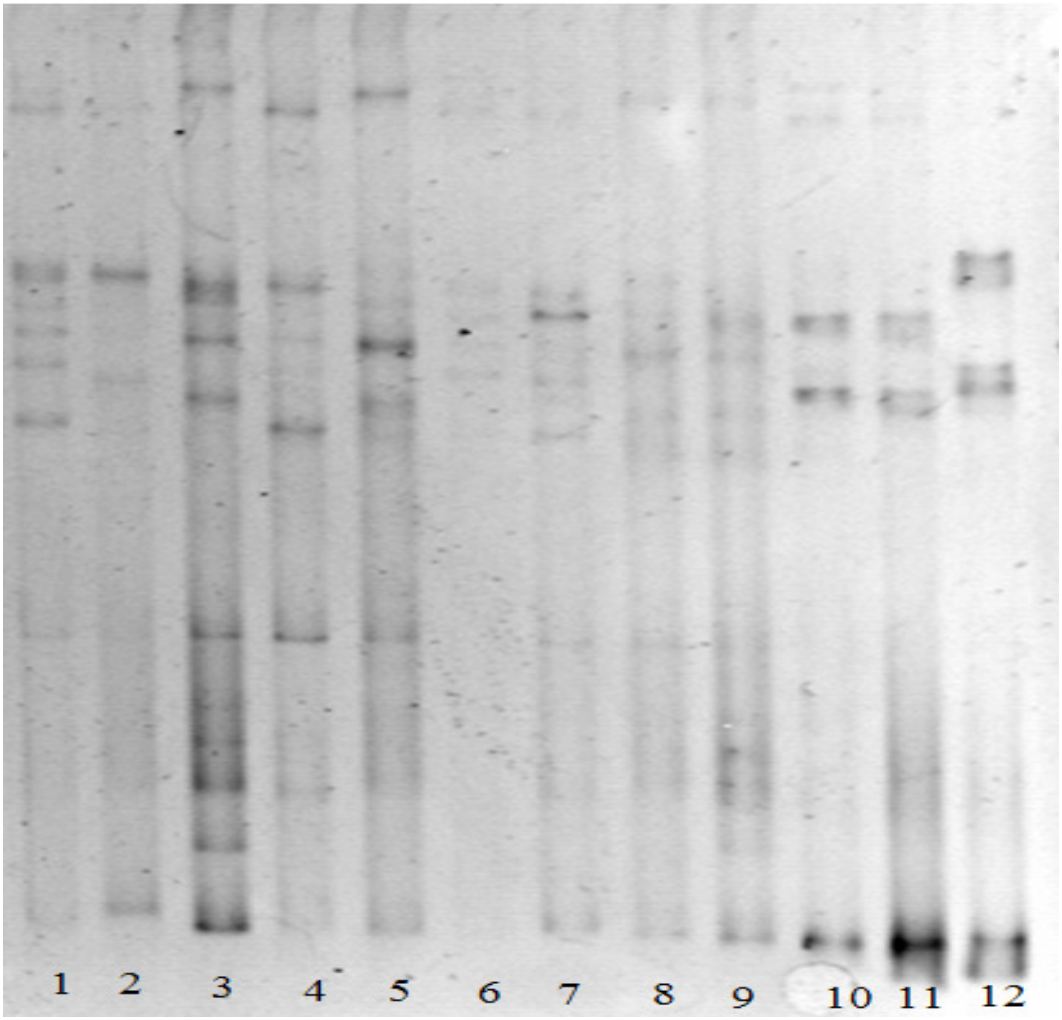

Figure 17 SSCP gel image of DRB patterns for zebras (*Ezh*) and horses (*Ec*) demonstrated in reverse contrast to the ethidium bromide stained gel: Lanes 1-12 represent *Ezh* 3183, *Ezh* 132, *Ec* 90, *Ezh* 510, *Ec* 2449, *Ezh* Solo, *Ezh* 509, *Ec* 190, *Ec* 172, *Ezh* 3861, *Ezh* 7041, *Ezh* 6819.

4.3.2.2 DQB

Single strand conformational polymorphism testing was conducted with 11 Hartmann's zebras and four horses. Polymorphic patterns were identified as shown in (**Figure 18**).

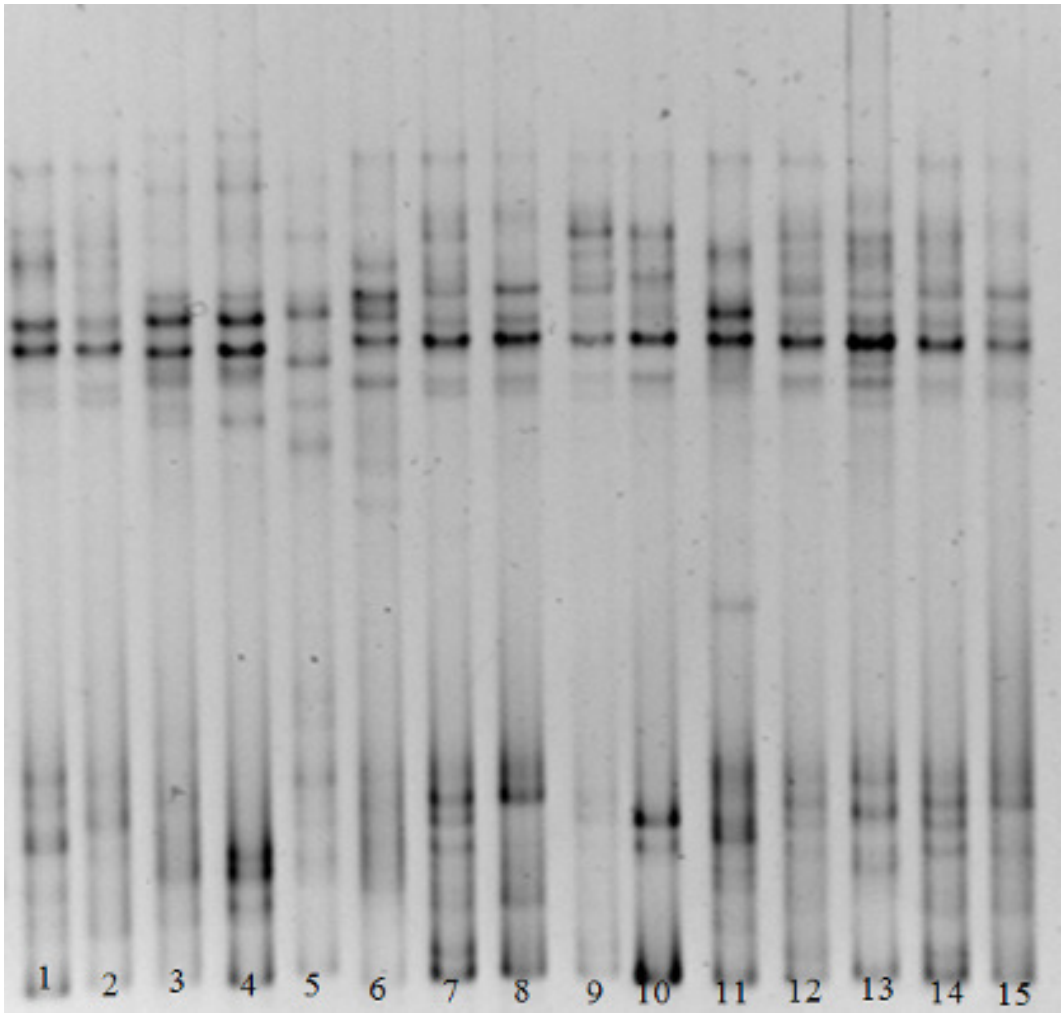

Figure 18 SSCP patterns for DQB in horses (*Ec*) and zebras (*Ezh*) demonstrated in reverse contrast to the ethidium bromide stained gel: Lanes 1-15 represent: *Ezh* 7040, *Ezh* Solo, *Ec* 172, *Ec* 90, *Ec* 190, *Ezh* 132, *Ezh* 509, *Ezh* 510, *Ezh* 3867, *Ezh* 7041, *Ezh* 6819, *Ezh* 454, *Ec* 2449, *Ezh* 3186, *Ezh* 10494.

Comparing gels from samples obtained from the zebra and the horse, the range and averages were similar but the zebras did exhibit slightly fewer bands than the domestic horses (**Table 7**). The identity of the DNA fragments as DRB products was confirmed by DNA sequencing for two individuals *Ezh* 509 and 510 (**Figure 15**) and the identity of DQB products was confirmed by DNA sequencing for three individuals *Ezh* 509, 510 and 132 (**Figure 16**).

Table 7 SSCP results from domestic horses *Equus caballus* (*Ec*) and Hartmann's zebra *Equus zebra hartmannae* (*Ezh*) for DRB and DQB.

Species	Range DRB: Numbers of SSCP bands	Ave. DRB	Range DQB: Numbers of SSCP bands	Ave. DQB
Ec	8-14	12.6	9-13	11.6
Ezh	6-13	10.2	6-12	10.6

4.4 DISCUSSION

Class I sequences demonstrated that among four clones tested for zebra 509, there were clearly three different class I sequences. Among five clones tested for zebra 1586 there were four different sequences. While one or two of the differences could be the result of cloning artifacts, there were multiple differences demonstrating that these were related but clearly different genes.

Based on projections from horses (Ellis *et al*. 1995; Tallmadge *et al*. 2005) and other species; (Aoyagi *et al*. 2002; Moon *et al*. 2005; Xu *et al*. 2007) and based on these preliminary studies, the class I genes are likely to be highly complex and require significantly greater work to characterize haplotypes in zebras than investigations of other MHC genes, such as class II loci.

In class II loci, the DRA gene is considered almost monomorphic in most species tested (Fraser *et al*. 1994; Zhou *et al*. 2007; Xu *et al*. 2007; Ballingall *et al*. 2010), but polymorphic in horses and other equids (Bailey 1994; Albright-Fraser *et al*. 1996; Brown *et al*. 2004; Luis *et al*. 2005; Arbanasic *et al*. 2009). The genetics of the DRA in zebras is unknown except that the methods used to characterize DNA sequences of horse exon two DRA are effective in other equine species.

All sequence data were consistent with a single locus for the DRA gene. Clones from three unrelated zebras were sequenced and none found to have more than two different sequences. If three different sequences were confirmed, this would suggest the existence of more than one locus. Subsequent sequencing of genomic DNA for five more zebras produced data consistent with a single locus. The sequencing data suggested that there were three alleles among the eight Hartmann's zebras (0501, 0601 and JBZ185) (**Table 6**).

Single strand conformational polymorphism typing led to the characterization of the alleles identified by Albright-Fraser *et al*. (1996) but did not allow clear assignment of genotypes for the

three different alleles. Specifically, separation was not obtained to distinguish between alleles 0501, 0601 and JBZ185. Therefore the alleles had to be distinguished by direct DNA sequencing. It can also be distinguished by another method, perhaps a method involving hybridization of target molecules.

As a result of not being polymorphic, the DRA gene cannot be used for typing in the zebra for differentiating between sarcoid and healthy zebra.

In the horse DRB has as many as three loci (Fraser & Bailey 1996) and possibly only two for some horses. In the Przewalski horse there appear to be only two loci (Hedrick *et al*. 1999). In the horse, this was too complex to type using SSCP and Fraser (1998) simply reported the existence of more than 23 alleles without being able to identify which loci were responsible. Diaz *et al*. (2001) confirmed multiple DRB copies in Argentine Creole horses, but were unable to identify specific alleles with specific loci.

In the absence of family data or of discrete molecular data for each individual, it is not possible to make a direct assignment of genotype from the phenotypes observed on the SSCP gels. However, one can compare the limited sequencing data with the SSCP phenotypes to determine whether the limited polymorphism found for sequencing corresponds to limited variation on the stained gel. We determined that zebra 510 is a heterozygote and that zebra 509 shares at least one allele. From the gel in **Figure 17** we can see that zebra 510 and zebra 509 each have four dark staining bands and additional faint bands. There is one pair of dark staining bands in zebra 510 that does not appear in zebra 509 and vice versa. Therefore, this is consistent with sharing one allele but each having a second different allele at that locus. Likewise, the phenotypic patterns observed when testing additional zebras are intermediate in complexity when compared to that observed for the horse / zebra DRA locus and the DRB locus in horses which is known to possess three loci (Fraser & Bailey 1996).

When examining the phenotypes for nine Hartmann's zebra, there are clear differences in phenotypes among them but there are also many bands shared between them. It is possible to score each zebra on each gel for the presence of fragments in common between different pairs of sets of zebra, reflecting the sharing of alleles at the DRB locus. This approach can be used to compare zebras with sarcoid tumours to those without. When a fragment is found more commonly among one group, it will be subjected to further study by cloning, sequencing and application of more specific typing of all zebras.

In horses the DQA locus was found to comprise a single locus in the MHC with at least 17 alleles and probably more (Szalai *et al*. 1994). Typing on SSCP resulted in patterns that reflected the great diversity of the locus but were relatively simple to interpret (Fraser 1998). Unfortunately, the primers that amplified the 2nd exon of DQA in horses were not effective in amplifying the product in zebras. Specifically, multiple products were produced and it was not possible to make the reaction more specific by increasing stringency. It is likely that success in typing for this locus would require the development of *Ezh* specific primers. Nevertheless, as a result of these difficulties this approach was not pursued, especially since the DRB and DQB appeared to be more effective.

In the horse Szalai *et al*. (1993) described a single locus with 13 alleles, for the DQB locus although Fraser (1998) found that the amplification strength of the alleles varied tremendously. Hořin and Matiašovic (2002) described two DQB loci and at least two groups of alleles were shown to exist. This data are consistent with the existence of two loci for DQB in these zebras. Zebra 132 had four different sequences among seven clones tested, zebra 509 had four sequences among nine clones tested and zebra 510 had three sequences among six clones tested. The data do not exclude the existence of more loci but are minimally compatible with two loci and complete heterozygosity for zebra 132 and zebra 509.

The DQB SSCP image (**Figure 18**) has two zones with DNA fragments. The pattern on the top is different from the pattern on the bottom so they represent entirely different gene products and not simply the positive and negative strand of DNA. This phenotypic appearance may reflect locus differences in the migration of allele fragments from the two (minimum) loci for DQB in zebras. Future genetic studies could be directed toward determining the relationship of alleles, loci and the SSCP pattern.

As noted from the sequencing it can be anticipated that DQB genes have at least two loci. This is reflected in the more complex SSCP phenotypic pattern observed when compared to the pattern for DRB (presumed one locus) shown above (**Figure 15**). As described for DRB, the bands can be scored and compared for the zebras on each gel and homologous and non-homologous fragments identified, reflecting similarities and difference in DQB MHC types between the individuals.

In conclusion, to establish the haplotypes in the zebra, class II MHC genes DRB and DQB could be used as a typing system in the sarcoid-affected zebra herds.

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CHAPTER FIVE:

MHC CLASS II VARIATION AMONG ZEBRAS WITH SARCOID TUMOURS

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ABSTRACT

The endangered Cape mountain zebra (*Equus zebra zebra*) are protected in a few small isolated populations in South African game parks. Because of the small numbers, the herds are prone to inbreeding and development of hereditary problems. In 1995, sarcoid lesions appeared on zebras in two parks, affecting up to 24% and 53% of the populations in each park. This is much higher than the less than 1% prevalence among horses. Certain major histocompatibility complex (MHC) haplotypes are associated with increased risk for sarcoid tumours in horses. This study was undertaken to investigate whether or not zebras in these parks had become inbred for the MHC region with increased prevalence of a haplotype, conferring increased risk for sarcoid tumours. Single strand conformational polymorphism was used to assess genetic variation at the MHC among these zebras. Twelve sarcoid affected and 12 controls from herds where the disease occurred as well as 10 controls from zebras in parks where no sarcoid has been observed before, were tested. The results demonstrated that genetic variation existed for the MHC class II genes in these zebras and no haplotype was found associated with the presence of sarcoid tumours.

Keywords: MHC, sarcoid, Cape mountain zebra, polymorphism

5.1 INTRODUCTION

The Cape mountain zebra (*Equus zebra zebra*) was one of the rarest mammals in the world (Penzhorn 2003) and classified as vulnerable on the International Union for the Conservation of Nature and Natural Resources (IUCN) Red List 2009 (http://www.iucnredlist.org). They were saved from the brink of extinction and are protected in small numbers in a few isolated populations in South African game parks in their natural habitat. Although the numbers slowly increased in recent years, the genetic diversity and the continued existence of Cape mountain zebras are of great concern as the uneven distribution of a few relatively large populations makes them still vulnerable (Novellie *et al*. 2002).

Cape mountain zebra herds in South Africa had been confined in fenced areas for many generations and the opportunity of high inbreeding as a result of the non-territorial social organization of Cape mountain zebra occurred (Penzhorn & Novellie, 1991). For instance the population increase to a high density in a short period of time and cases have been reported where a young stallion became the herd stallion of his maternal herd (Penzhorn & Novellie, 1991). In 1995, equine sarcoid-like lesions (24.7% of animals) appeared in Cape mountain zebras from the Gariep Dam Nature Reserve (Free State Province) (Nel *et al*. 2006) and in 1998 in the Bontebok National Park (Western Cape Province) (53% of animals) (Lange 2004). An isolated case of a zebra that was euthanized due to the severity of sarcoid lesions was reported in 2004 in the Mountain Zebra National Park (Eastern Cape Province) (personal communication: Dr Dave Zimmerman).

The term “equine sarcoid” was first used by Jackson in South Africa in 1936 to describe a distinctive fibroblastic neoplasm occurring in the skin of horses, donkeys and mules (Jackson, 1936). It was also used to distinguish it from papilloma, fibroma and fibrosarcoma. Equine sarcoid is a locally aggressive, non-regressing, fibroblastic skin tumour and the most commonly found dermatological skin lesion in equidae (Goodrich *et al*. 1998). A survey among Swiss Warmblood and Freiberger horses found sarcoid tumors among 0.07% and 0.4%, respectively (Dubath M-L 1986). Bovine papillomavirus types 1 and 2 have been detected in sarcoid tumours of horses, donkeys, mules (Lancaster *et al.* 1977; Angelos *et al*. 1991; Bloch *et al*. 1994; Nasir *et al*. 1997; Nasir & Reid 1999; Chambers *et al*. 2003) and more recently in zebras in captivity. (Löhr *et al*. 2005). Sarcoid tumours are associated with BPV but the tumour does not produce infectious virions (Amtmann *et al*. 1980; Lancaster, 1981).

Sarcoid susceptibility in horses was found associated with MHC class I or class II antigens in family studies (Marti *et al*. 1996). In particular, horses with MHC class II haplotypes possessing the ELA-W13 serological specificity had an increased risk for sarcoid tumours (Lazary *et al*. 1985). Associations between class II MHC genes and papillomavirus-induced tumors have also been described in other species. In humans, papilloma virus-induced squamous cell carcinoma of the cervix is more common in women carrying the HLA-DQW3 class II MHC allele (Wank & Thomssen 1991). Apple *et al*. (1994) found a papilloma virus type-specific association between HLA DR-DQ haplotypes in this carcinoma of the cervix. Shope papillomavirus, which induce the development of tumors in the rabbit, are associated with class II MHC genes (Han *et al*. 1992).

The development of a typing system specific to these zebras was created and characterization of the genes using a molecular approach was described in the previous chapter. Single strand conformational polymorphism reproducibly detects genetic variation in DNA and has been used effectively to investigate MHC class II among horses (Fraser, 1998). It has also proved to work well (Chapter 4) to determine the extent of genetic variation for the MHC class II genes of zebras.

The recent increased prevalence of sarcoid tumors among the zebras in Bontebok National Park and Gariep Dam Nature Reserve may be the result of the closed herds becoming inbred for an MHC haplotype that confers increased susceptibility to sarcoid tumors. If this is the case, then one would expect reduced genetic variation of the MHC relative to other parks.

Therefore, the purpose of the study was to determine whether or not the high prevalence of sarcoid among Cape mountain zebra is associated with a MHC haplotype.

5.2 MATERIALS AND METHODS

5.2.1 Study population and sample collection

For comparison of zebras with sarcoid tumors to those without, samples were collected from zebras at game parks in South Africa and testing conducted at the University of Pretoria (UP). To implement the typing system, blood samples were collected in EDTA-buffered tubes by venipuncture of the jugular vein and stored at 4 °C from a total of 34 zebras located in different national parks in South Africa. Blood were collected from affected Cape mountain zebra (*Equus zebra zebra*) in the Gariep Dam Nature Reserve (Free State Province) (n=18), Bontebok National Park (Western Cape Province) (n=4) and Mountain Zebra National Park (n=2). Blood were collected from zebras in parks where no sarcoid tumours have been observed before, from

Hartmann's mountain zebra (*Equus zebra hartmannae*) (n=3) in the Augrabies National Park (Northern Cape Province) and the Cederberg Wilderness Area (Western Cape Province) (n=2); Burchell's zebra (*Equus quagga burchelli*) (n=2) in the Karoo National Park (Western Cape Province) and Cape mountain zebra in the Karoo National Park (n=3). The age of the animals varied between 3-15 years. These samples were processed and the typing system applied in the Molecular Laboratory, Department of Veterinary Tropical Diseases, UP, Onderstepoort, South Africa.

5.2.2 DNA extraction

DNA was extracted from 200 µl of blood collected from the above mentioned zebras 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.

5.2.3 Conventional PCR amplification of the second exon class II

Class II DRB, and DQB genes were PCR amplified using the primers and conditions as described in Chapter 4. The amplified regions lie within the second exon of both genes and amplicons were used for SSCP typing.

5.2.4 Molecular cloning and sequencing

The primer pairs for MHC class II DQB and DBR that were used to amplify the genomic sequences were described in **Table 5** (Chapter 4). The amplicons were purified, cloned into the pGEM®-T 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 analysed on an ABI 3100 sequencer. Sequencing data were assembled and edited with the GAP4 program of the Staden package (version 1.6.0 for Windows) and aligned with published sequences of related genera using ClustalX (version 1.81 for Windows). A BLAST search was performed using the Blastn algorithm to confirm the identity of the DNA fragments as DRB and DQB products.

5.2.5 MHC Typing: Single-Strand Conformational Polymorphism Analysis

Analysis was based on the SSCP method originally described by Orita *et al*. (1989). The SSCP gels consisted of a 10% w/v acrylamide:bis-acrylamide (37.5:1) (BioRad, South Africa) 0.5X TBE buffer (44.5mM boric acid, 1mM Na_2-EDTA, pH=8.4) (Qiagen, Southern Cross Biotechnology, South Africa), 0.093% w/v ammonium persulphate (MERCK, South Africa) and 0.08% v/v N,N,N',N'-tetramethylethylenediamine (TEMED) (MERCK, South Africa). The samples consisted of PCR products from genomic DNA as well as cloned PCR products.

Samples (8 µl water + 3 µl loading dye + 2 µl PCR products) were denatured in a heating block at 90 °C for 10 minutes and put immediately on ice until loaded. The gels were electrophoresed in 0.5X TBE buffer on a Protean II xi cell gel apparatus (BioRad Laboratories Inc., Melville, NY) at 100 volts for 20 hours at 20 °C. The SSCP gel was visualized by staining for 10 minutes in a 0.5X TBE with 1 ul of a 1 mg/ml stock solution of ethidium bromide, and photographed under UV trans-illumination. The gels were scored by counting the number of fragments visible on the gel.

5.3 RESULTS

5.3.1 Amplification of MHC DNA fragments

Amplifications of Cape mountain zebra DNA using primers outlined in **Table 5** (Chapter 4) were successful for DQB and DRB and produced DNA fragments the same size (210 and 276 bp) in Cape mountain zebras (*Ezz*) as in horses and Hartmann's zebras (*Ezh*).

5.3.2 Comparison of SSCP DRB and DQB profiles for zebras with and without sarcoid tumours

5.3.2.1 DRB

None of the bands on the gels occurred exclusively among zebras with sarcoid tumours or zebras without sarcoid tumours.

Among the profiles of the 13 zebras illustrated in **Figure 19** there were eight different phenotypes, five among the zebras with sarcoid tumours and three among the zebras without sarcoid tumours. Of the 11 bands scored on the gel, eight were polymorphic. No association occurred exclusively among the zebras with sarcoid tumours or those without sarcoid tumours.

5.3.2.2 DQB

As in DRB none of the bands on the gels occurred exclusively among zebras with sarcoid tumours or zebras without sarcoid tumours. Among the profiles of the eight zebras illustrated in **Figure 20** there were seven different phenotypes, five among the zebras with sarcoid tumours and two among the zebras without sarcoid tumours. Of the 12 bands scored on the gel, 10 were polymorphic. No association occurred exclusively among the zebras with sarcoid tumours or those without.

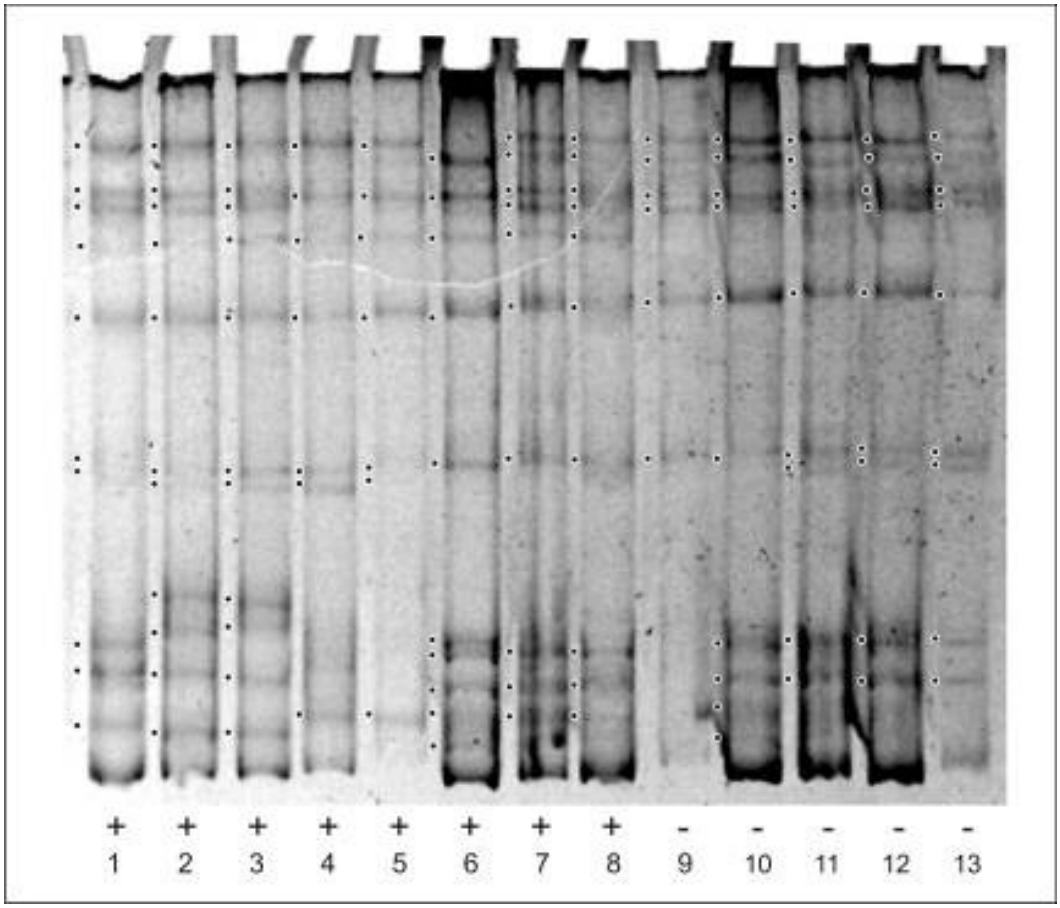


Figure 19 DRB SSCP patterns from genomic DNA amplifications for different zebra demonstrated in reverse contrast to the ethidium bromide stained gel: Lane 1-8 represent *Equus zebra zebra* (*Ezz*) numbers 1, 5, 7, 32, 35, 79, 82, 83, all sarcoid positive, Lane 9-13: *Ezz* 102, *Equus zebra hartmannae* (*Ezh*) 130, *Equus quagga burchelli* (*Eqb*) 158, *Eqb*160, *Ezh* 176, all sarcoid negative.

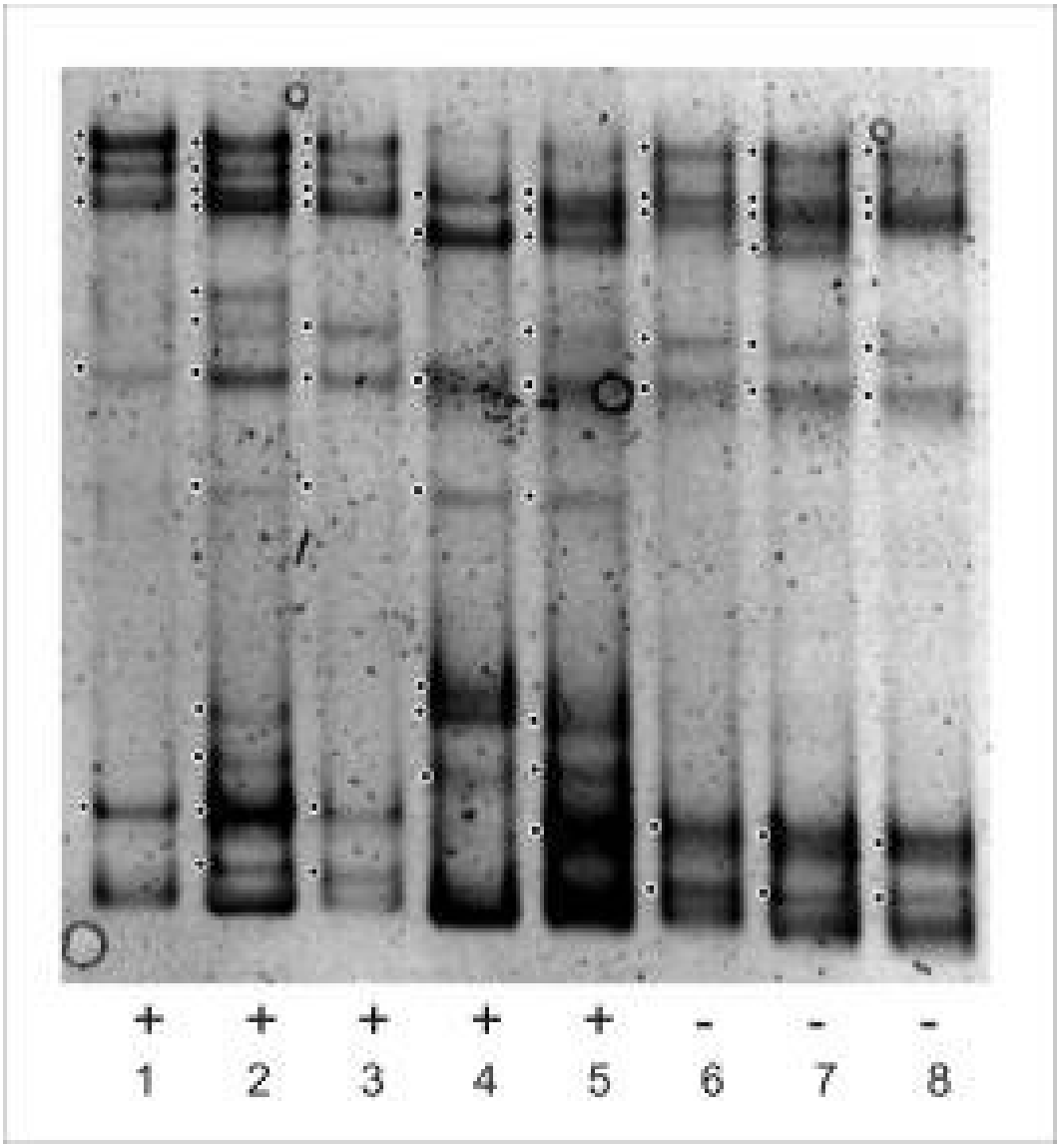
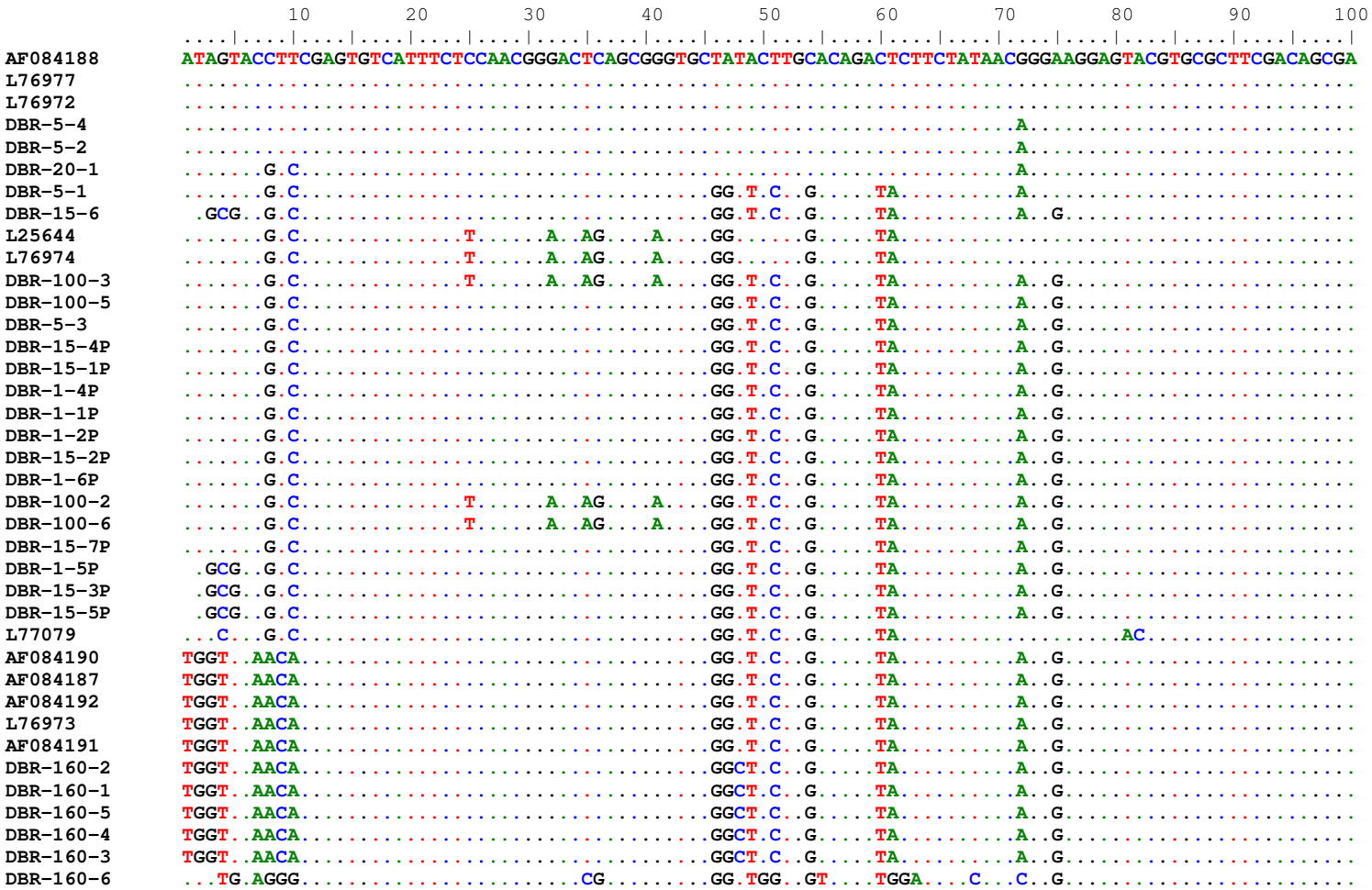
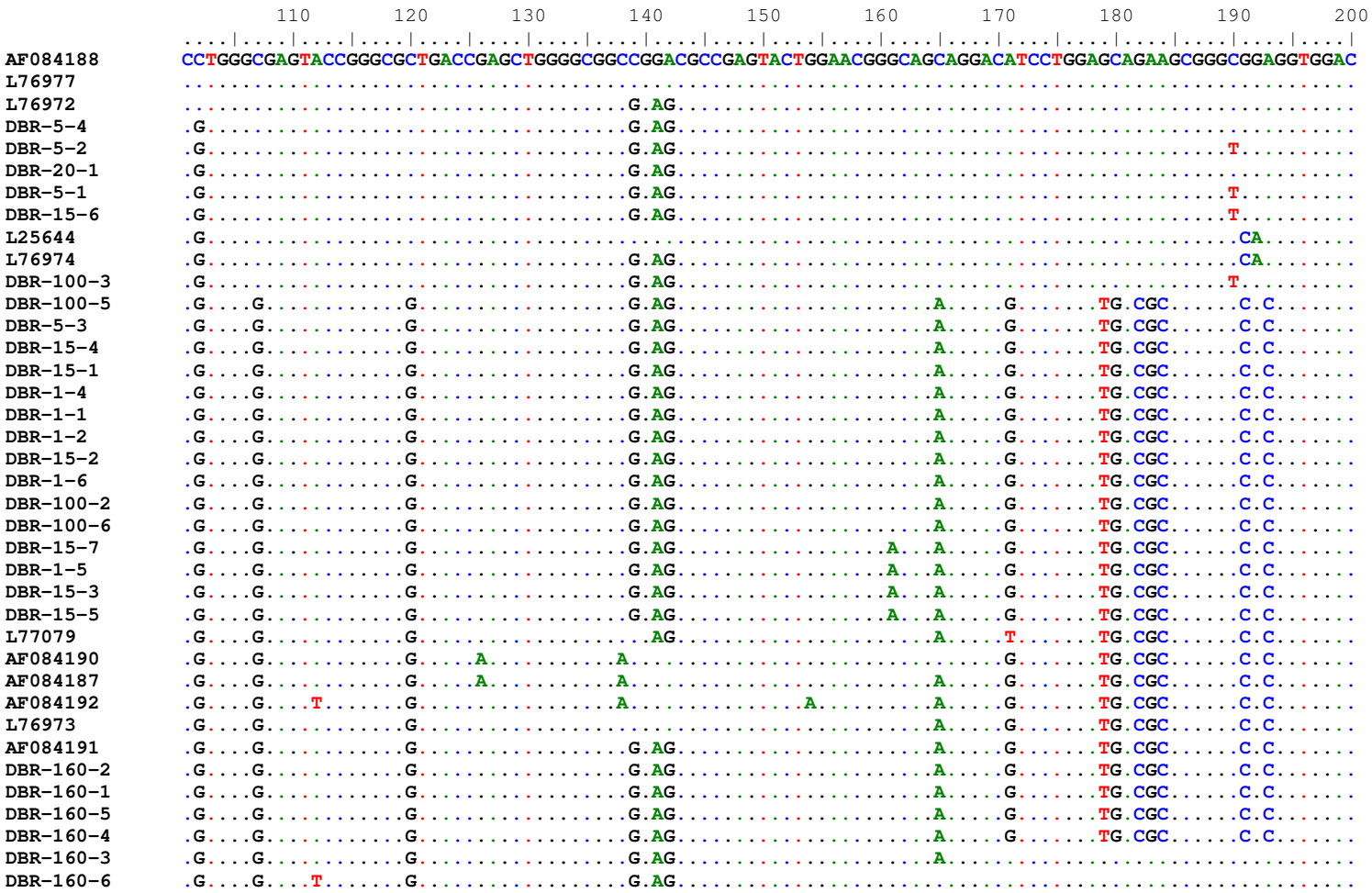


Figure 20 DQB SSCP patterns from genomic DNA amplifications for zebra with and without sarcoid demonstrated in reverse contrast to the ethidium bromide stained gel: Lane 1-5 sarcoid positive zebra *Ezz* numbers 1, 7, 79, 91, 97. Lanes 6-8 sarcoid negative zebra *Ezz* numbers 100, 109, 112.

5.3.3 Sequencing

DRB second exon sequences for clones from sarcoid affected zebra (*Ezz* 1, 5, 15, 20) and sarcoid unaffected zebra *Ezz* 100 and *Equus zebra burchelli* (*Ezb*) 160 are tabulated in **Figure 21** compared to equine DRB sequences obtained from GenBank. Sequences from 17 clones tested from four sarcoid affected zebras and 10 clones tested from two healthy zebras showed genetic diversion among sarcoid-affected as well as healthy zebras. BLAST results showed high similarity with *Equus przewalski* class II antigen DBR gene accession numbers (AF084177, AF084178, AF084190, AF08491, AF08492).


10 20 30 40 50 60 70 80 90 100
AF084188 ATAGTACCTTCGAGTGTCATTTCTCCAACGGGACTCAGCGGGTGCTATACTTGCACAGACTCTTCTATAACGGGAAGGAGTACGTGCGCTTCGACAGCGA
L76977
L76972
DBR-5-4
DBR-5-2
DBR-20-1
DBR-5-1
DBR-15-6
L25644
L76974
DBR-100-3
DBR-100-5
DBR-5-3
DBR-15-4P
DBR-15-1P
DBR-1-4P
DBR-1-1P
DBR-1-2P
DBR-15-2P
DBR-1-6P
DBR-100-2
DBR-100-6
DBR-15-7P
DBR-1-5P
DBR-15-3P
DBR-15-5P
L77079
AF084190
AF084187
AF084192
L76973
AF084191
DBR-160-2
DBR-160-1
DBR-160-5
DBR-160-4
DBR-160-3
DBR-160-6


110 120 130 140 150 160 170 180 190 200
AF084188 CCTGGGCGAGTACCGGGCGCTGACCGAGCTGGGGCGGCCGGACGCCGAGTACTGGAACGGGCAGCAGGACATCCTGGAGCAGAAGCGGGCGGAGGTGGAC
L76977
L76972
DBR-5-4
DBR-5-2
DBR-20-1
DBR-5-1
DBR-15-6
L25644
L76974
DBR-100-3
DBR-100-5
DBR-5-3
DBR-15-4
DBR-15-1
DBR-1-4
DBR-1-1
DBR-1-2
DBR-15-2
DBR-1-6
DBR-100-2
DBR-100-6
DBR-15-7
DBR-1-5
DBR-15-3
DBR-15-5
L77079
AF084190
AF084187
AF084192
L76973
AF084191
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DBR-160-5
DBR-160-4
DBR-160-3
DBR-160-6

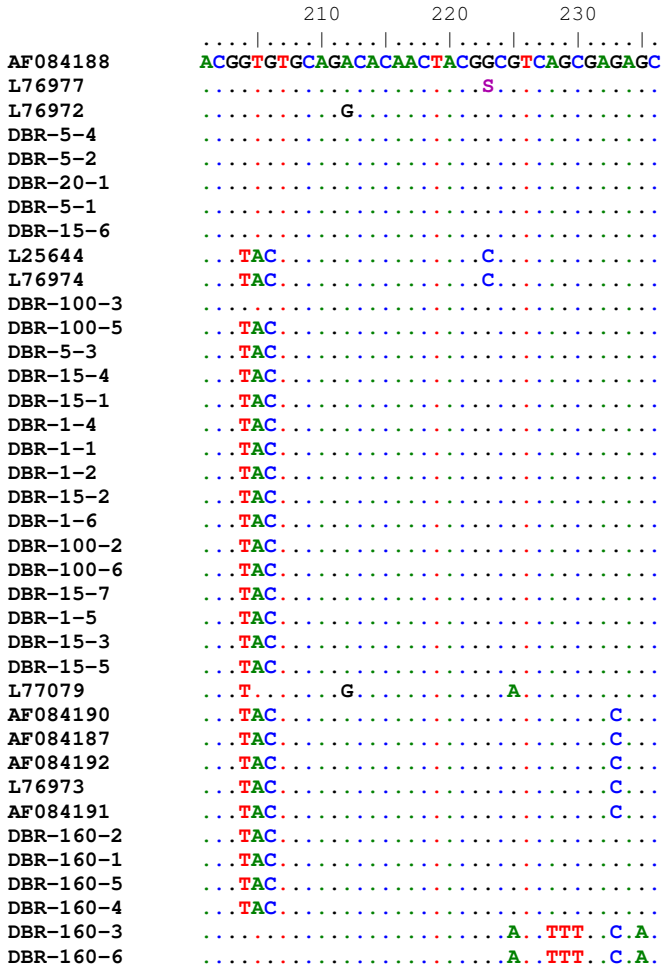
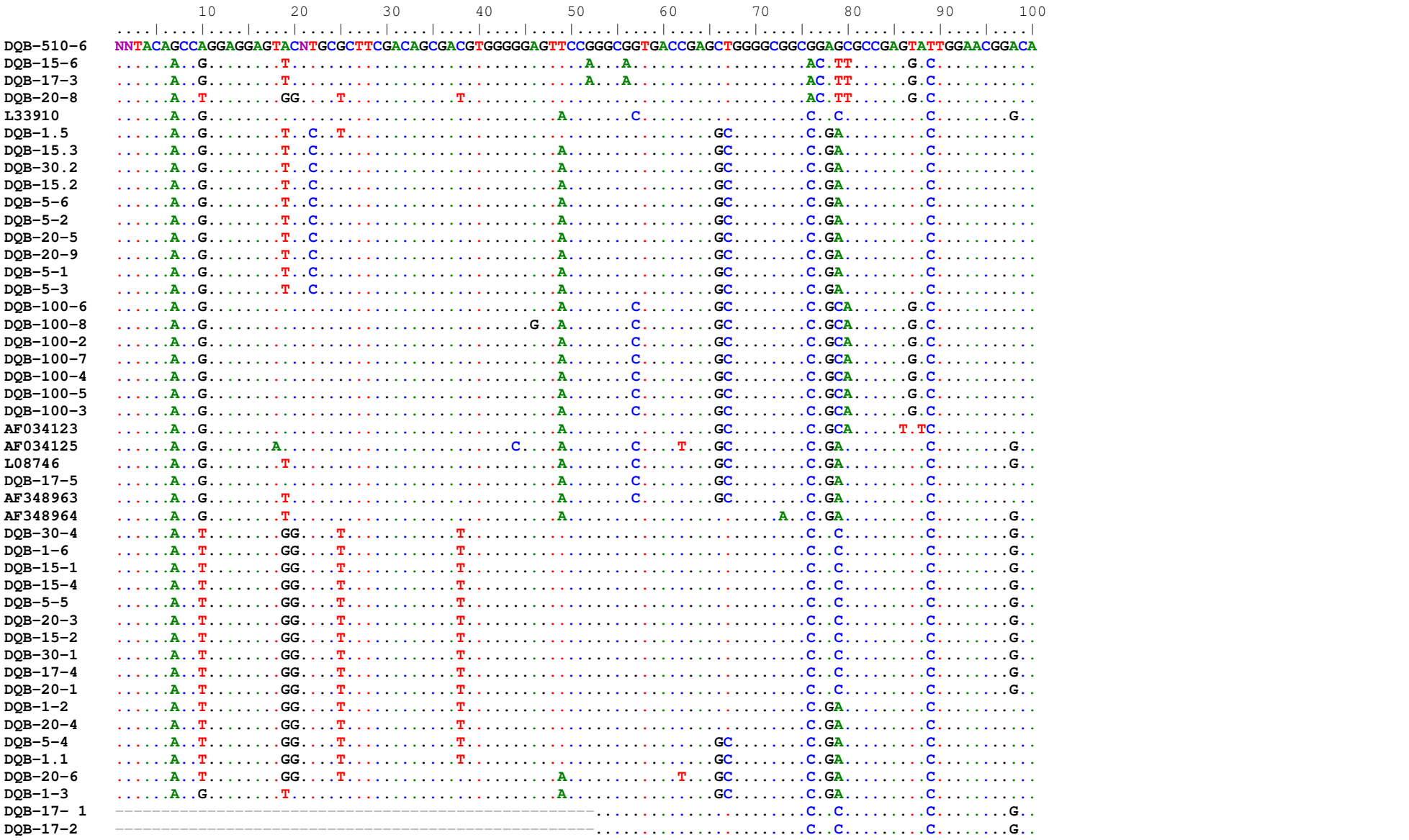


Figure 21 Alignment of nucleotide sequences for the 2nd exon of MHC class II DBR clones (designated "DRB-animal number-clone number") from *Ezz* 1, 5, 15, 20, 100, and *Ezh* 160. (Ezz 1, 5, 15, 20 are sarcoid positive) *Eqpz* accession numbers AF084187; AF084188; AF084190; AF184191; AF084192; and *Ec* accession numbers L25644; L76972; L76973; L76974; L76977; L77079 are used as references.


10 20 30 40 50 60 70 80 90 100
DQB-510-6 NNTACAGCCAGGAGGAGTACNTGCGCTTCGACAGCGACGTGGGGGAGTTCCGGGCGGTGACCGAGCTGGGGCGGCGGAGCGCCGAGTATTGGAACGGACA
DQB-15-6
DQB-17-3
DQB-20-8
L33910
DQB-1.5
DQB-15.3
DQB-30.2
DQB-15.2
DQB-5-6
DQB-5-2
DQB-20-5
DQB-20-9
DQB-5-1
DQB-5-3
DQB-100-6
DQB-100-8
DQB-100-2
DQB-100-7
DQB-100-4
DQB-100-5
DQB-100-3
AF034123
AF034125
L08746
DQB-17-5
AF348963
AF348964
DQB-30-4
DQB-1-6
DQB-15-1
DQB-15-4
DQB-5-5
DQB-20-3
DQB-15-2
DQB-30-1
DQB-17-4
DQB-20-1
DQB-1-2
DQB-20-4
DQB-5-4
DQB-1.1
DQB-20-6
DQB-1-3
DQB-17- 1
DQB-17-2

```
                  110       120
          ....|....|....|....|....|..
DQB-510-6 GAAGGACATCCTGGAGGAGTACCGGGC
DQB-15-6  .......G........C..A.G.....
DQB-17-3  .......G........C..A.G.....
DQB-20-8  .......G........C..A.G.....
L33910    .......G........C..ACG.....
DQB-1-5   ..............AG.ACG.....
DQB-15-3  ..............AG.ACG.....
DQB-30-2  ..............AG.ACG.....
DQB-15-2  ..............AG.ACG.....
DQB-5-6   ..............AG.ACG.....
DQB-5-2   ..............AG.ACG.....
DQB-20-5  ..............AG.ACG.....
DQB-20-9  ..............AG.ACG.....
DQB-5-1   ..............AG.ACG.....
DQB-5-3   ..............AG.ACG.....
DQB-100-6 ..............AG.ACGT....
DQB-100-8 ..............AG.ACGT....
DQB-100-2 ..............AG.ACGT....
DQB-100-7 ..............AG.ACGT....
DQB-100-4 ..............AG.ACGT....
DQB-100-5 ..............AG.ACGT....
DQB-100-3 ..............AG.ACGT....
AF034123  ..............CAG.ACGT....
AF034125  ..............C..ACG.....
L08746    .......T........C..ACG.....
DQB-17-5  ..............A..ACG.....
AF348963  ..............CG.ACG.....
AF348964  ..............A..ACGT....
DQB-30-4  ..............CG.GTG.....
DQB-1-6   ..............CG.GTG.....
DQB-15.1  ..............CG.GTG.....
DQB-15.4  ..............CG.GTG.....
DQB-5-5   ..............CG.GTG.....
DQB-20-3  ..............CG.GTG.....
DQB-15-2  ..............CG.GTG.....
DQB-30-1  ..............CG.GTG.....
DQB-17-4  ..............CG.GTG.....
DQB-20-1  ..............AG.ACG.....
DQB-1-2   ..............AG.ACG.....
DQB-20-4  ..............AG.ACG.....
DQB-5-4   ..............AG.ACG.....
DQB-1-1   ..............AG.ACG.....
DQB-20-6  ..............AG.ACG.....
DQB-1-3   ..............AG.ACG.....
DQB-17-1  ..............CG.GTG.....
DQB-17-2  ..............CG.GTG.....
```

Figure 22 Alignment of nucleotide sequences for the 2nd exon of MHC class II DQB clones (designated "DQB-animal number-clone number") from *Ezz* 1, 5, 15, 20, 30, (sarcoid positive) and *Ezz* 17, 100 and *Ezh* 510 (sarcoid negative). *Ec* accession numbers L33910; L08746; AF348963; AF348964; *Ea* accession numbers AF034123; AF034125 are used as references.

DQB second exon sequences for clones from sarcoid affected zebra (*Ezz* 1, 5, 15, 20, 30) and sarcoid unaffected zebra *Ezz* 17 and 100 are tabulated in **Figure 22** compared to equine sequences obtained from GenBank to identify the DNA fragments as DQB products. Sequences from 26 clones from seven sarcoid affected zebras and 10 clones from two healthy zebras showed genetic diversion among sarcoid as well as healthy zebras. BLAST results showed a high similarity (~93%) with MHC class II antigen (ELA-DQB) gene, *Ea* reference number (U31775) and *Ec* reference number (AF348963).

5.4 DISCUSSION

The system developed for typing MHC in Hartmann's zebras by using SSCP worked well for the Cape mountain zebras. PCR primers developed for amplification of the horse MHC genes also worked well for amplifying the Cape mountain zebras MHC gene. DNA sequencing confirmed the identity of the DNA fragments as being MHC class II gene products and confirmed that the DNA fragments under investigation were for MHC-DQB and MHC-DRB based on homology to the DNA sequence of comparable horse genes.

The hypothesis that the herd was inbred and the increase of sarcoid tumours was due to loss of variation in the MHC region could not be confirmed. However, the number of DNA fragments seen among the different groups of zebras in South Africa was similar to that observed for zoo maintained zebras from the United States and comparable to that seen for domestic horses. Different electrophoresis apparatuses were used in Kentucky, where the typing system was developed, and South Africa, where the sarcoid affected herds were investigated. The unit used in Kentucky was longer, allowing slightly more resolution.

Whilst recombination is thought to be reduced in MHC of many species, it could still occur, and in the absence of the identification of the causative mutation it could be that the markers used will show different frequencies rather than categorical differences. The allele frequencies of the two populations could have been statistically compared to see if any of the MHC alleles are significantly compared with sarcoid development. The age range of the zebras (3-12 years) might however change the allele distribution, as negative animals might develop sarcoids in the future. The sample size used is too small for this type of experiment, and it is rare for susceptible loci that one allele is observed exclusively in the sarcoid-affected or control population.

Despite the lower resolution for SSCP typing, variation was readily found for both DRB and DQB among the zebras affected with sarcoid tumours. Furthermore, there were no fragments found which were unique to either the affected or unaffected zebras. These results do not support a genetic influence of the MHC for the very high occurrence of sarcoid tumors among zebra in these parks and the hypothesis that the zebras become inbred for the MHC, leading to homozygosity for a susceptible haplotype, clearly did not occur. It does not exclude the fact that the MHC can be involved, there could be a haplotype that has become more frequent and that SSCP, being an insensitive test, did not discern it from others. The cause for the high appearance of sarcoid in these parks should also be investigated elsewhere, perhaps by studies of environmental factors causing immunosuppression and thereby stimulating growth of sarcoid tumours among zebras.

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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.

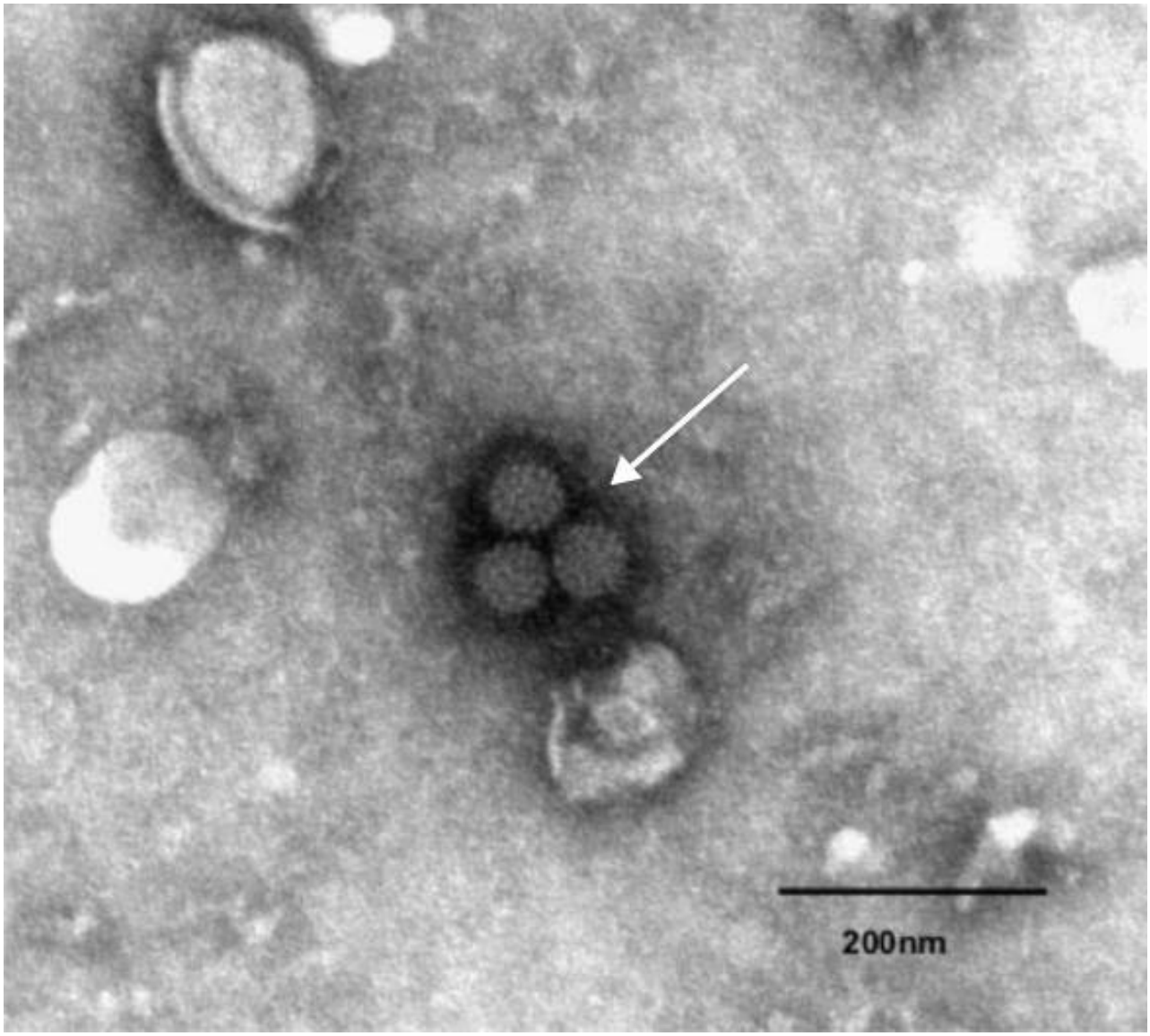

Figure 25 Negatively-stained intranuclear papillomavirus particles in an unfixed sample from the skin of the sable cow.

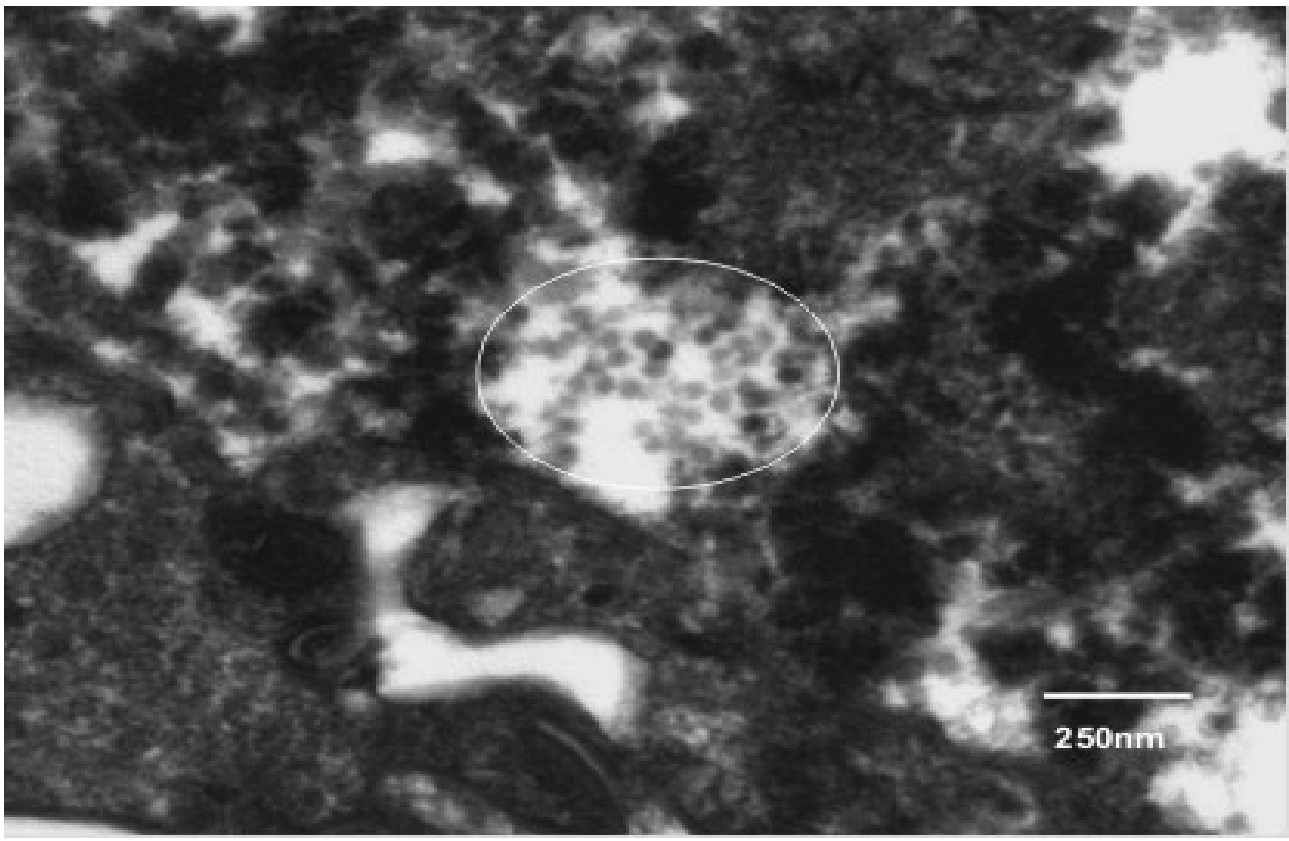

Figure 26 Intranuclear papillomavirus (encircled) particles stained positively with uranil-acetate in ultra thin sections of wax-retrieved epoxy sections of the sable cow skin.

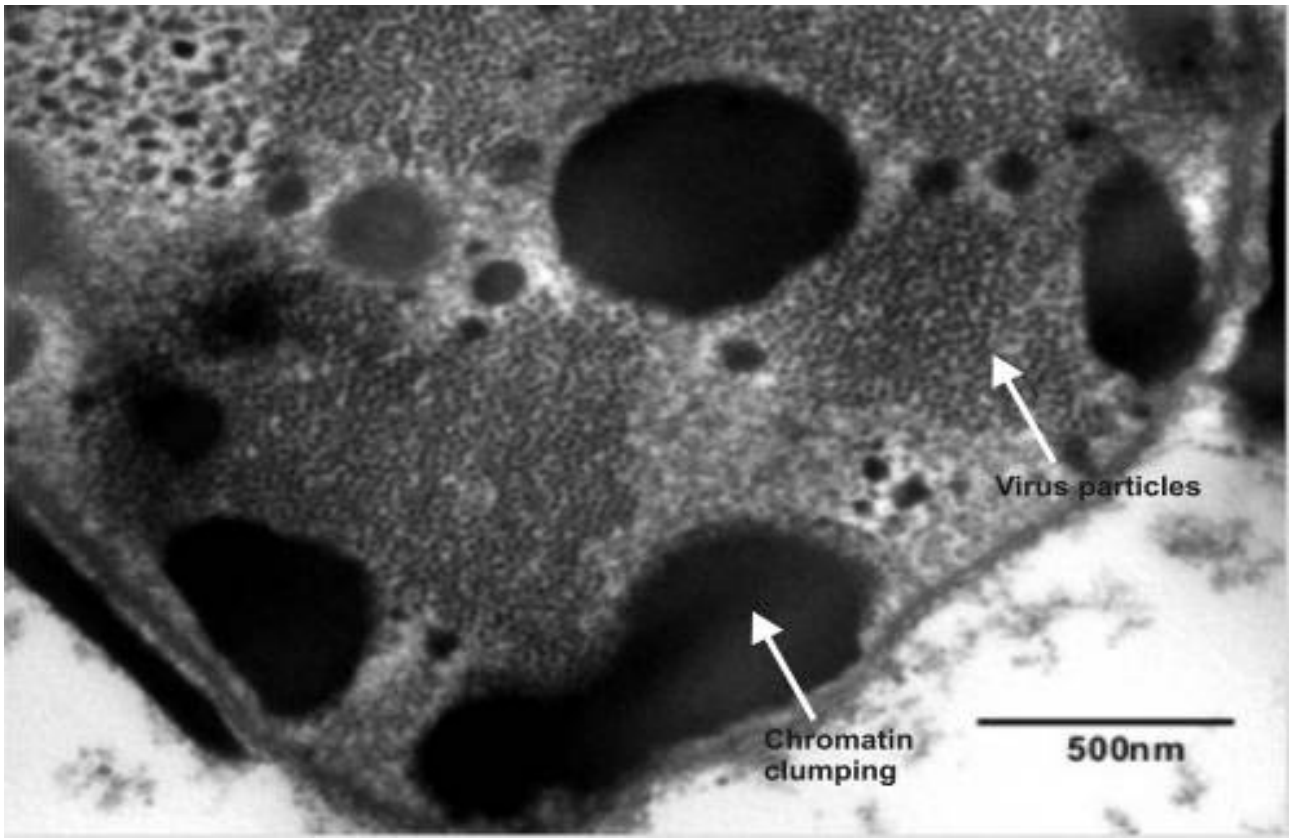

Figure 27 Numerous intranuclear papillomavirus particles in sections of formalin-fixed skin of Giraffe 1. Note nuclear chromatin clumping.

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.

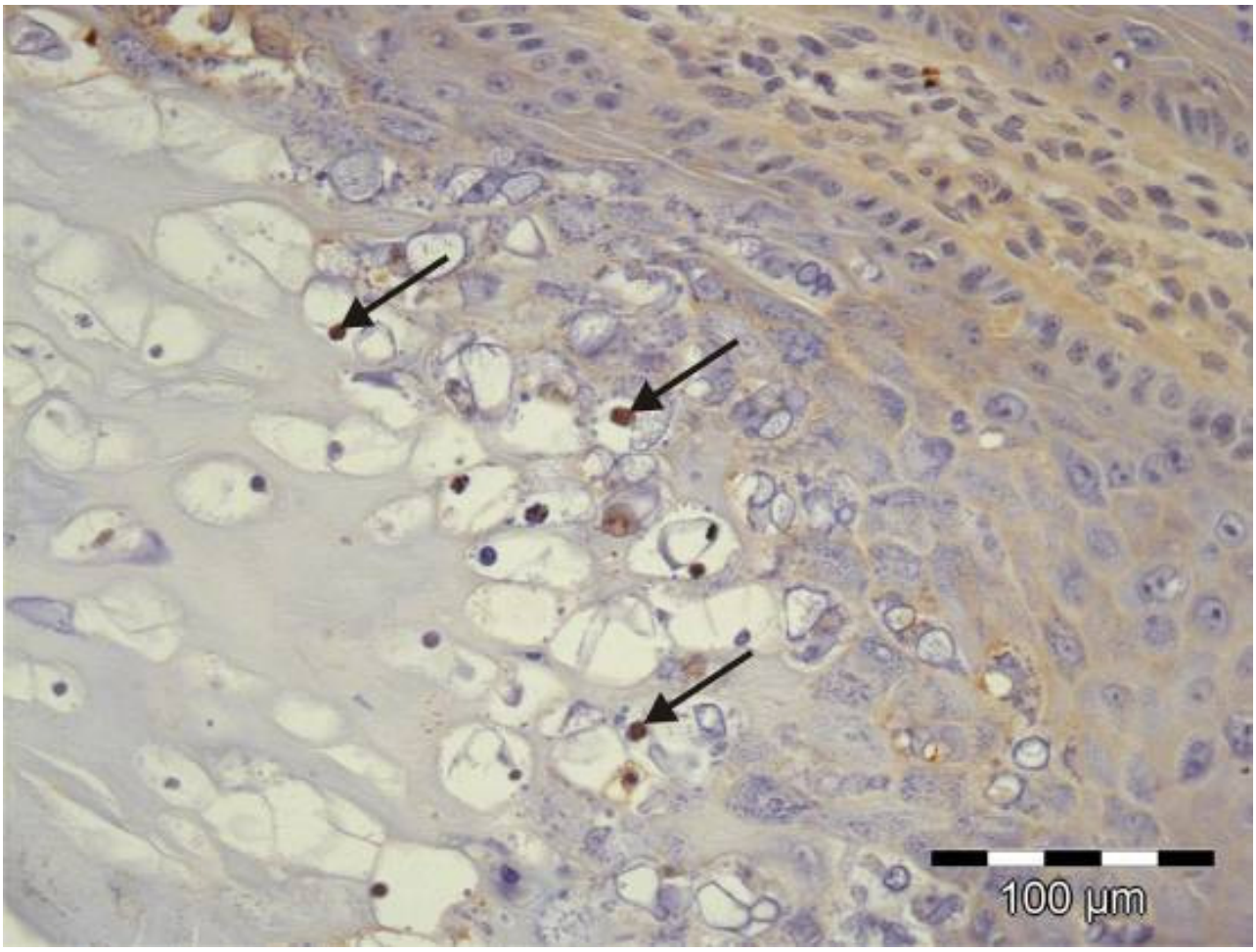

Figure 28 Lesion from sable showing occasional papillomavirus-positive *stratum granulosum* nuclei on immunohistochemistry stained with immunoperoxidase stain.

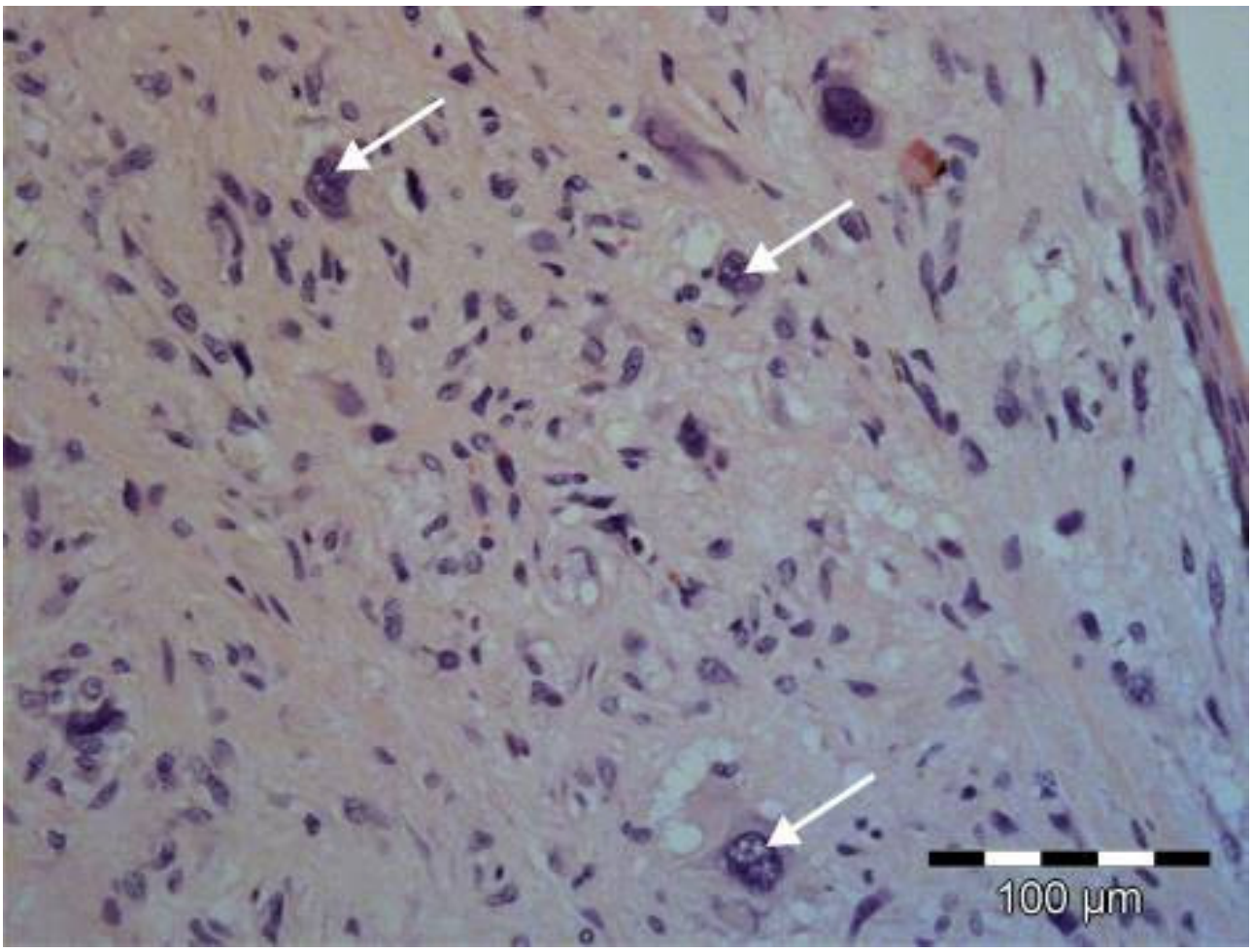

Figure 29 Giraffe 2: subepidermal sarcoid-like lesion stained with haematoxylin and eosin showing multifocal bizarre anaplastic fibroblasts (arrows).

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.

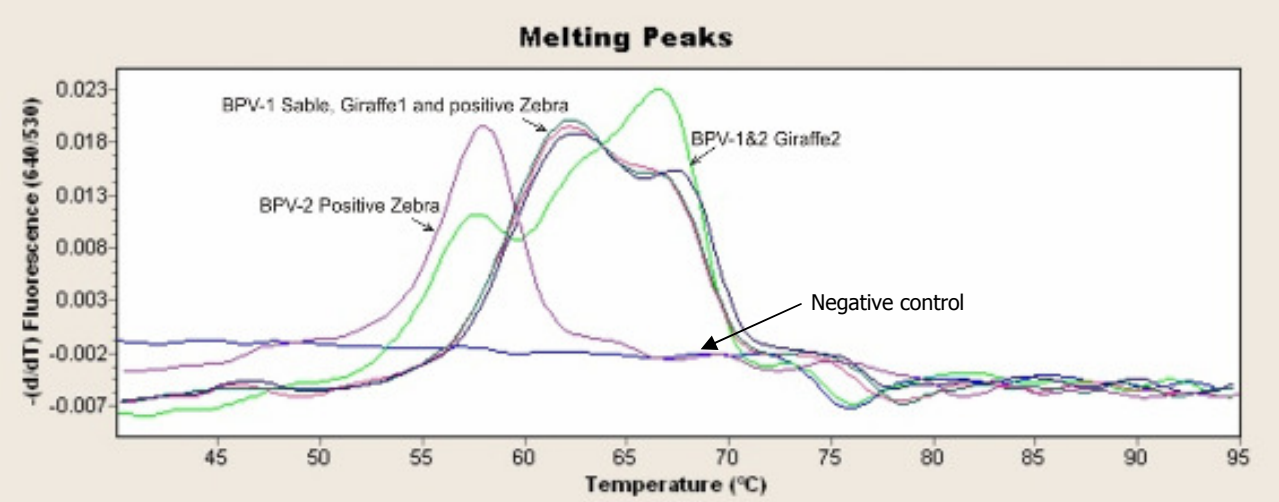

Figure 30 Real-time PCR results of the sable and Giraffe 1 demonstrating BPV-1 DNA, and showing an increase in fluorescence at 62.90±1.24 °C in both. The positive BPV-1 control zebra shows the same pattern while the control zebra for BPV-2 reveals an increased fluorescence at 58.48±0.61 °C. Giraffe 2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

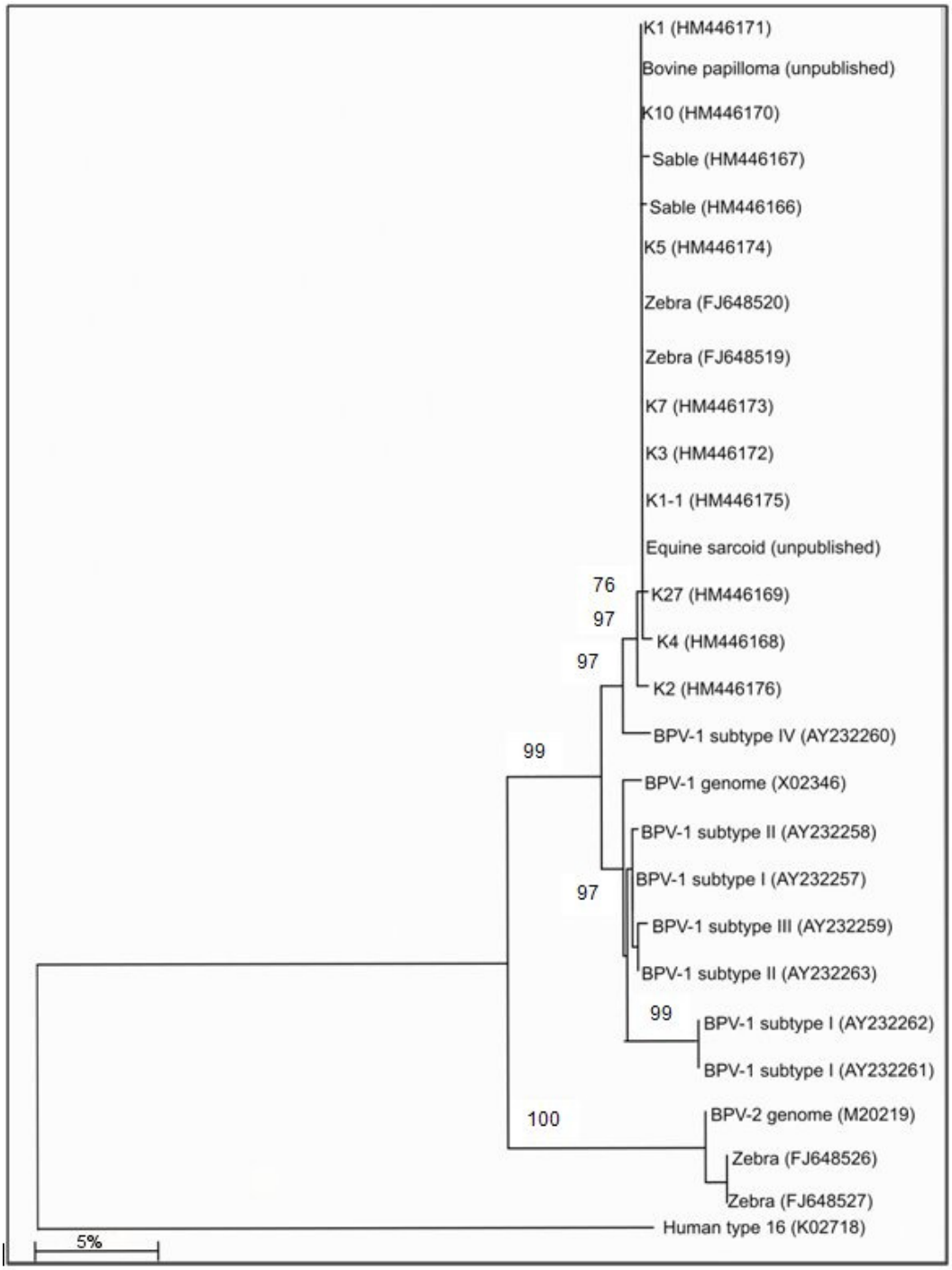

Figure 31 Results of the neighbour-joining tree of BPV E2E5 encoding gene showing the phylogenetic relationship of sequences compared to sequences of BPV-1 and BPV-2 obtained from GenBank.

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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CHAPTER SEVEN:

CONCLUSION

The occurrence of sarcoid in the endangered Cape mountain zebras in the Bontebok National Park and Gariep Dam Nature Reserve in South Africa is a reality which needs urgent attention. A very high incidence of this condition was reported (24% and 53%) of the populations of this vulnerable species. The zebras were severely stressed and were unsightly for the many tourists visiting these parks. Their condition deteriorated as result of the growths and they become debilitated and died. The study was performed to investigate if bovine papillomvirus (BPV) is associated with sarcoids in the free-roaming zebras, to develop a real-time polymerase chain reaction (PCR) diagnostic method to detect and distinguish between BPV-1 and -2, to study the phylogenetic relationships isolated from sarcoids in the zebras in comparison with other equidae species and to determine whether or not the high prevalence of sarcoids in these herds is associated with a major histocompatibility complex (MHC) haplotype. Furthermore, to determine ultrastructurally if BPV is present in the papillomas in the skin of the giraffe and a sable antelope, and if so, to detect and distinguish between BPV-1 and -2 using the real-time PCR method.

Although BPV-1 and -2 are associated with sarcoids in horses, donkeys, and mules as well as in captive zebras their presence in free-roaming zebras has not been previously described. Cattle, which might be a source of the virus, are not associated with the parks and, although neighbouring farms might previously have harboured cattle, both of the affected parks are not situated in cattle farming districts and it is highly unlikely that large herds of cattle ever previously occurred in the regions concerned. The Bontebok National Park, however, did incorporate an area which was earlier used as a racehorse-track, where from time to time horses were grouped together. The Bontebok National Park was proclaimed in 1931 and the Gariep Dam Nature reserve in 1972. Since then zebras have been free-roaming and there has been no human intervention.

Histologically, the lesions in the zebras exhibited the identical features to those of sarcoids in the horse but, as in the horse, no intact virus particles could be demonstrated electron microscopically. However BPV DNA was demonstrated by PCR and RFLP. It was shown that both BPV-1 and BPV-2 DNA are indeed present in sarcoids in these zebra. Single BPV-1 or BPV-2 infections as well as mixed infections occurred in individual animals. The virus DNA was only detected from

sarcoid tissue and was not found in the blood or unaffected skin of the animals when examined by conventional PCR. The reason why the autogenous vaccine used to combat the disease had a higher therapeutic rate in the one park could be explained as it was shown that the vaccine used there contained both BPV-1 and -2 whereas in the other park, the vaccine used only contained BPV-1 and several cases did not respond to treatment.

A 637 bp product was cloned and sequenced to prove that the product is indeed bovine papillomavirus in which, in a broad selection of papillomavirusses compiled in a phylogenetic tree, the phylogenetic position of BPV-1 and -2 is well nested within the delta-epsilon-zeta papillomavirus superclade which includes papillomaviruses infecting several different hosts. This confirms the theory that BPV plays an important role in the aetiology and pathogenesis of equine (which includes those of the zebras in this investigation) sarcoids.

When the evolutionary relationships of the E5 gene sequences obtained from zebra BPV were determined the zebra sequences clustered not only with BPV-1 but also with BPV-2 as sister clades to other related equid sequences. The age for the most recent common ancestor for BPV-1 variants was estimated to be 1.4 Mya, while that for BPV-2 variants was estimated to be 0.55 Mya. The age for the most recent common ancestor for BPV-1 and -2 has been estimated to be 5.34 Mya. This can be used in further studies to explain the virus-host switch and adaptations to the new host. Further studies should, however, rather include sequences of the more variable upstream regulatory region, which is positioned between the L1 and L6 genes, where more BPV variants have been described.

A rapid, sensitive and reliable real-time PCR assay to detect and distinguish between BPV-1 and -2 infections in the zebras was developed. Using this sensitive method it was found that the virus DNA is also present in the normal skin and in the blood of healthy animals even from parks where sarcoids do not occur. The concentration of the virus found varied, being the highest in the sarcoid tissue, followed by the skin and lastly the blood, and was evident by the different threshold values of the amplification curves.

The virus is therefore present in healthy animals. The infection is however, latent and the animals show no clinical signs of disease. The immune system of an infected animal plays an important role in the outcome of papillomavirus infections as immune suppression and physical trauma can reactivate latent asymptomatic papillomavirus infections and clinical signs appear. A systematic

investigation of the immune response in animals suffering from sarcoid should yield results of great interest.

The reason why certain animals succumb to the disease was investigated by investigating their genetic background. The small populations all originated from the Mountain Zebra National Park where the zebras are protected and are progeny of the original 11 individual animals. It has been suggested that in the horse a hereditary predisposition exists, which was established by the strong association between prevalence of sarcoid and genes in or near the equine MHC. The precise cause for the association of MHC class II in horses with sarcoid tumours, however, has never been determined. The ELA DW13 serological specificity, associated with some sarcoid cases, is simply a genetic marker for a haplotype with multiple loci of class II genes.

A typing system to investigate the MHC haplotypes in the zebra was developed using SSCP. No differences in phenotypes were observed between the zebras in the affected parks based on the presence of sarcoid tumours. The zebras in these parks, although inbred herds, had a variation at the MHC for class II (DRB and DQB) genes which give them enough genetic diversity and protection against a variety of pathogens. The increased occurrence of sarcoid tumours in the zebras in Bontebok National Park and Gariep Dam Nature Reserve is not due to inbreeding and homozygosity at the MHC since the zebras do show variation. From the results obtained in this study no genetic marker could be identified for sarcoids in the zebra. With new analytical methods, for instance, single nucleotide polymorphism, a causative mutation might be found, where regions of the genome with and without disease are compared.

The two parks in which the affected zebras occur are the smallest parks in which Cape mountain zebras are present. The habitat in both parks is not ideal for the species. In Bontebok National Park there is a general conflict of interest: that of managing the herbivores *versus* that of flora conservation. Two red-listed herbivore species the bontebok (*Damaliscus pygarus dorcas*) and the Cape mountain zebra (*Equus zebra zebra*) are protected here. The red hartebeest (*Alcelaphus buselaphus*) also occurs here. The suitability of the habitat is ideal for bontebok conservation but is only marginal for zebras. The vegetation consists mainly of fynbos and renosterveld. The fynbos biome includes both fynbos and renosterveld, in which the shrubs have small hard leaves but there are very few trees and grasses. In renosterveld, a shrub known as climber's friend or "steekbossie" (*Cliffortia ruscifolia)* occurs. This is a prickly bush with very sharp leaves which can easily traumatise the skin. Renosterbos (*Elytropappus rhinocerotis*) is characteristically dominant and is very unpalatable. All the shrubs in the renosterveld are characterized by possessing small, tough,

grey leaves. In the past, grasses were abundant in renosterveld but this led to their being overgrazed and replacement by shrubs; therefore grasses are uncommon today.

The zebras in Gariep Dam Nature Reserve also compete with other herbivores for the available grazing, which is classified as eastern mixed Nama Karoo. The vegetation consists of grassy dwarf shrubs interspersed with tassel bristlegrass (*Aristida congesta*) and Lehmann's lovegrass (*Eriocephalus ericoides*). The seeds of both these grass species possess very sharp awns which could cause superficial wounds to the skin. This reserve is situated outside the normal habitat of the Cape mountain zebra which favour mountainous areas containing their preferred type of grazing.

Tick infestations on the zebras in both parks also occur. The ticks traumatise the skin, especially its ventral aspects. It is felt that their role as possible vectors in the development of the disease should also be investigated to determine if the papillomavirus is transgenic in the ticks and what role they could have in the host-switch of the papillomavirus. The possible role of other vectors, such as flies, particularly, those which bite, and oxpeckers (*Buphagus* species) could play a role and their importance should also be investigated. They could play a role in virus host switch and virus evolution upon host switching.

South Africa is at times subjected, both generally and regionally, to severe droughts which could aggravate some of the the above conditions leading to stress in the animals and hence immunosuppression.

In two other species of wildlife, a giraffe and a sable antelope, papillomavirus was detected electron microscopically in cutaneous fibropapillomas. Histopathologically, the lesions in both species of animal showed features similar to those of equine sarcoid, but those in the giraffe showed histopathological evidence of malignant fibroblast pleomorphism. Since one of the giraffes tested in this study did not reveal any evidence of the presence of virus particles histologically, immunohistochemiscally or ultra structurally, it is considered that it was the end-point of BPV infection. Infection with the virus was, however, still detected by real-time PCR which is capable of detecting 1.5 gene copies of the virus. Clinical manifestation of a latent infection with papillomavirus in these animals may have been provoked by stress. In the case of the sable antelope, this could have been due to the fact that it was being kept in a habitat to which it was not accustomed and which could have been exacerbated by transport and resultant immunosuppression. In the case of the giraffes, one can speculate that drought conditions and the resultant high tannin content of the *Acacia*-trees, as a result of overbrowsing, as the latter are their preferred diet, could

act as a co-factor in BPV-associated carcinogenesis as occurs in cattle following bracken fern (*Pteridium aquilinum*) ingestion. This hypothesis requires investigation. No co-factor has as yet been identified for BPV-associated fibropapillomas or sarcoids.

In summary this was the first study demonstrating the presence of BPV-1 and -2 in sarcoid tissue of free-roaming zebra as well as the informative finding that BPV also occur in the skin and blood of clinical healthy animals, even in areas where sarcoid has never been observed. From this study, it is clear that inbreeding leading to homozygosity for a sarcoid susceptible haplotype is not the cause of sarcoid development although it does not exclude the possibility that the MHC is not involved. Animals succumb to the disease when they become immune suppressed and the virus overcome the immune response of the host. Game park officials should recognize stress-causing factors in game and act accordingly to prevent or minimize the disease.