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.

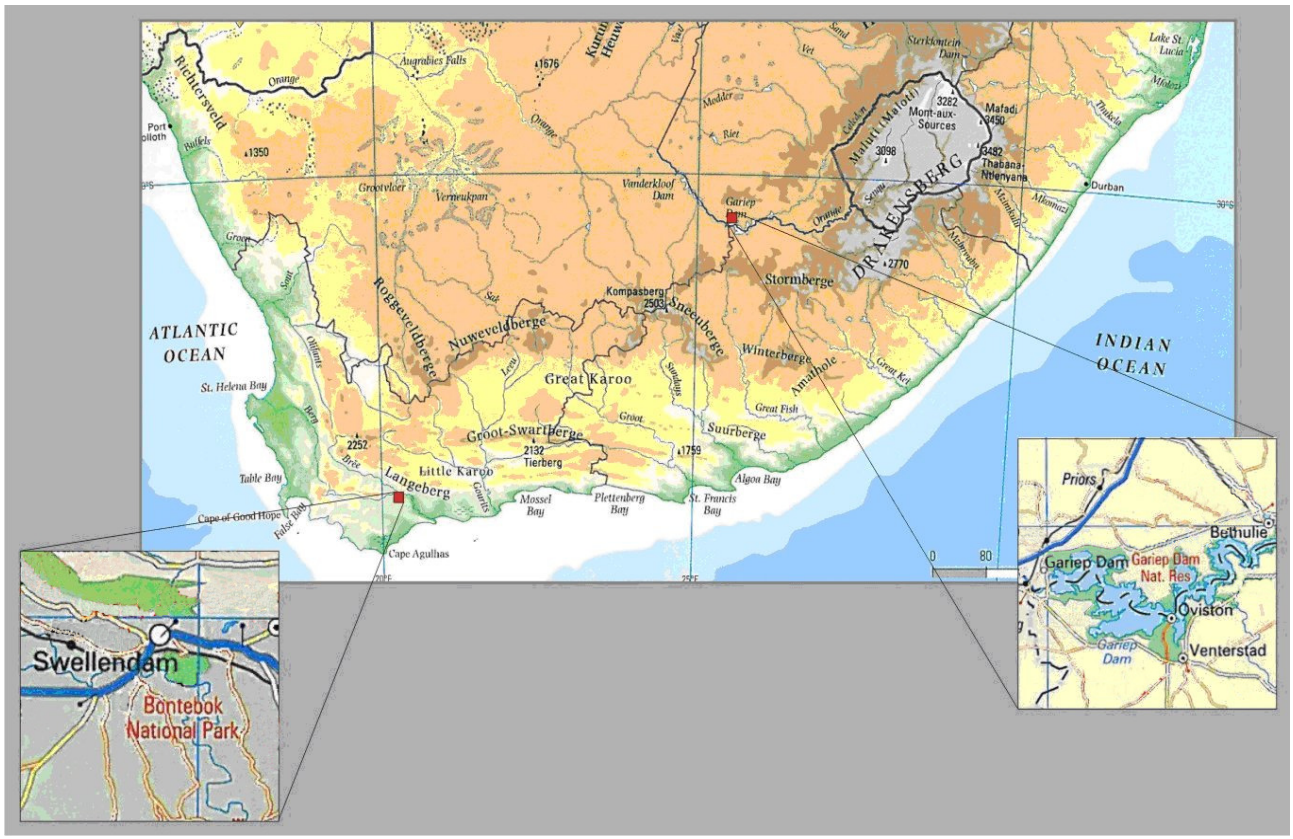

Figure 1 Geographical map of southern Africa showing the location of Gariep Dam Nature Reserve in the Free State and Bontebok National Park in the Western Cape Province. (Adapted from: MapStudio: Southern East Africa Road Atlas and Oxford Secondary Atlas for South Africa. Wiegrand P, (ed).

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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