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.

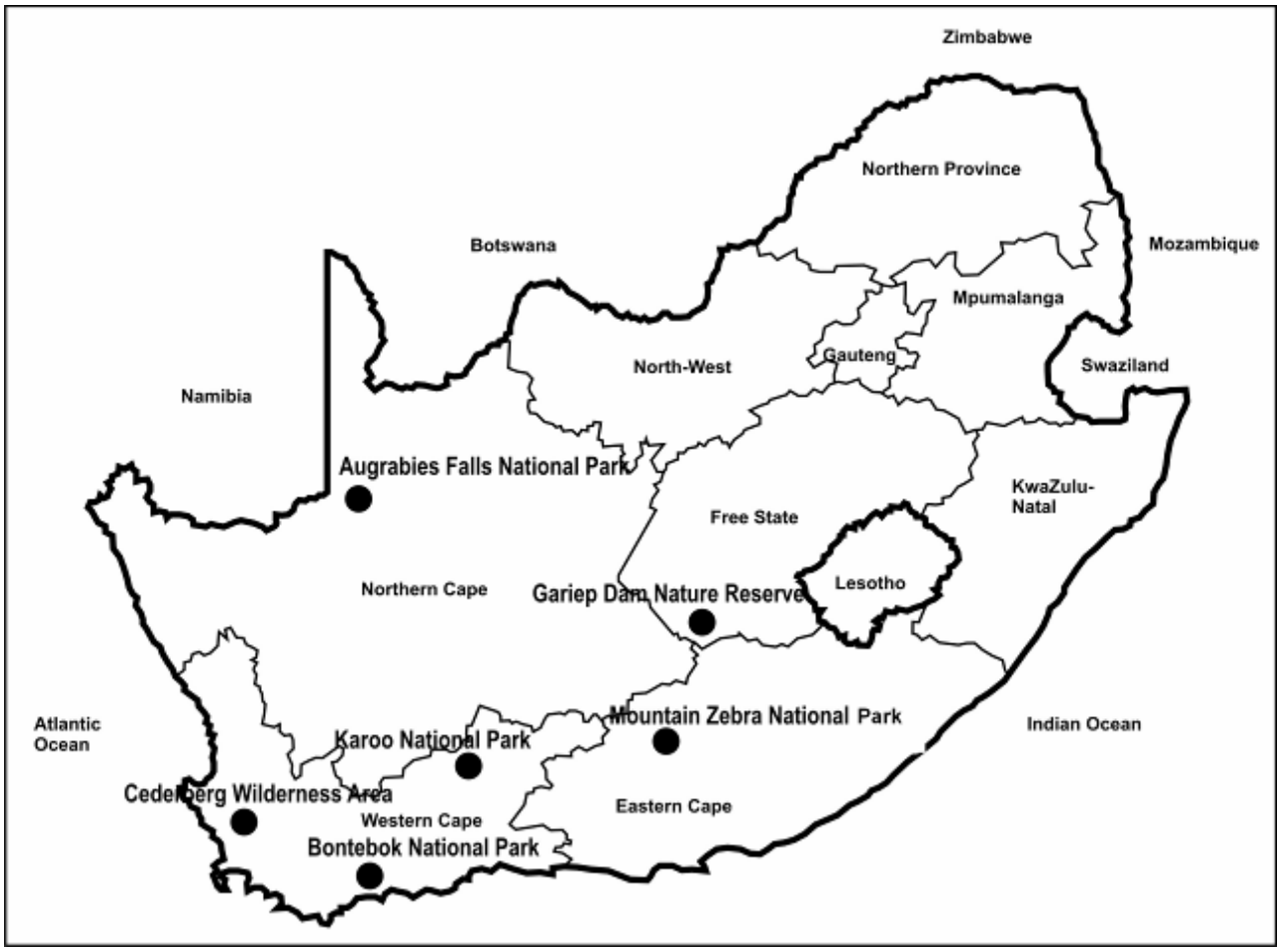

Figure 4 Map of South Africa illustrating the different areas where zebras have been sampled

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**).

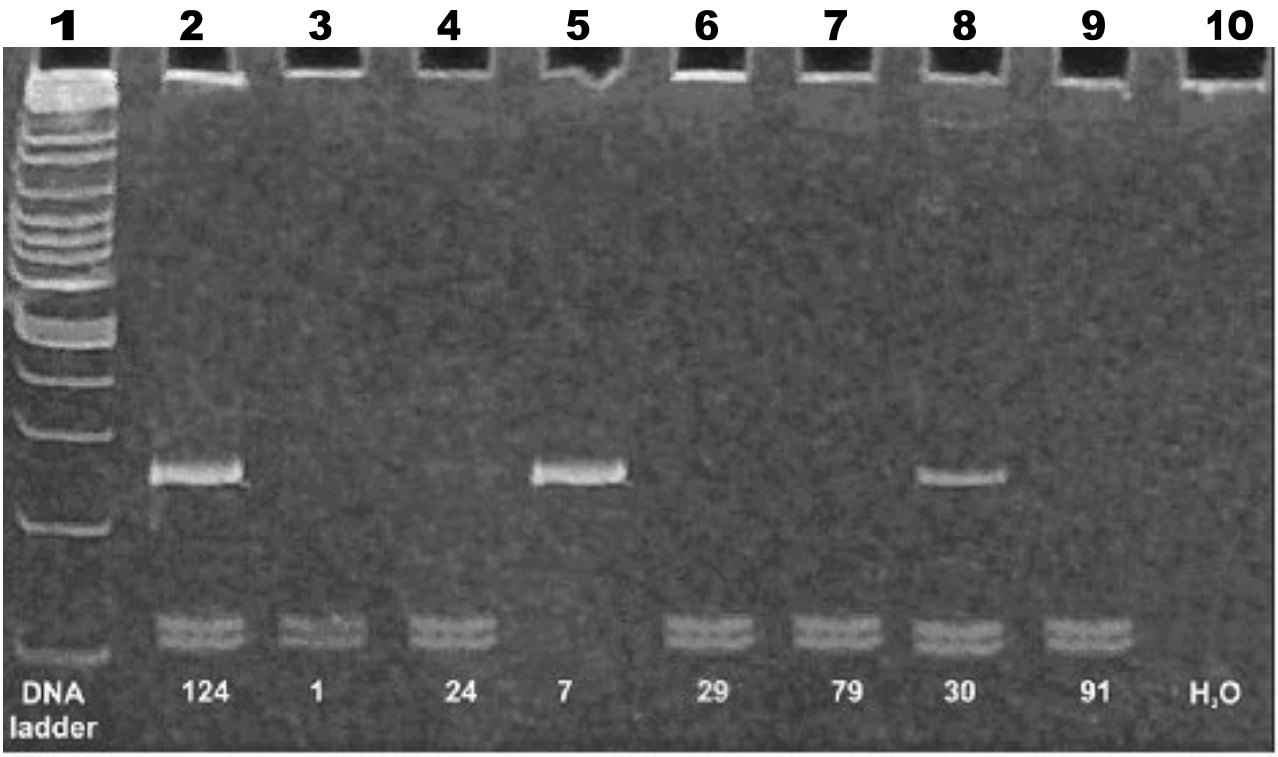

Figure 5 Polyacrylamide gel showing *Bst*XI restriction enzyme digestion of the PCR amplified E5 ORF of zebra sarcoid tumours. Lane 1=100 bp ladder, Lane 2 & 8=samples demonstrating both BPV-1 and BPV-2 infection, Lane 3, 4, 6, 7, 9=BPV1 infection, Lane 5=BPV 2 infection. Lane 10=negative control.

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.

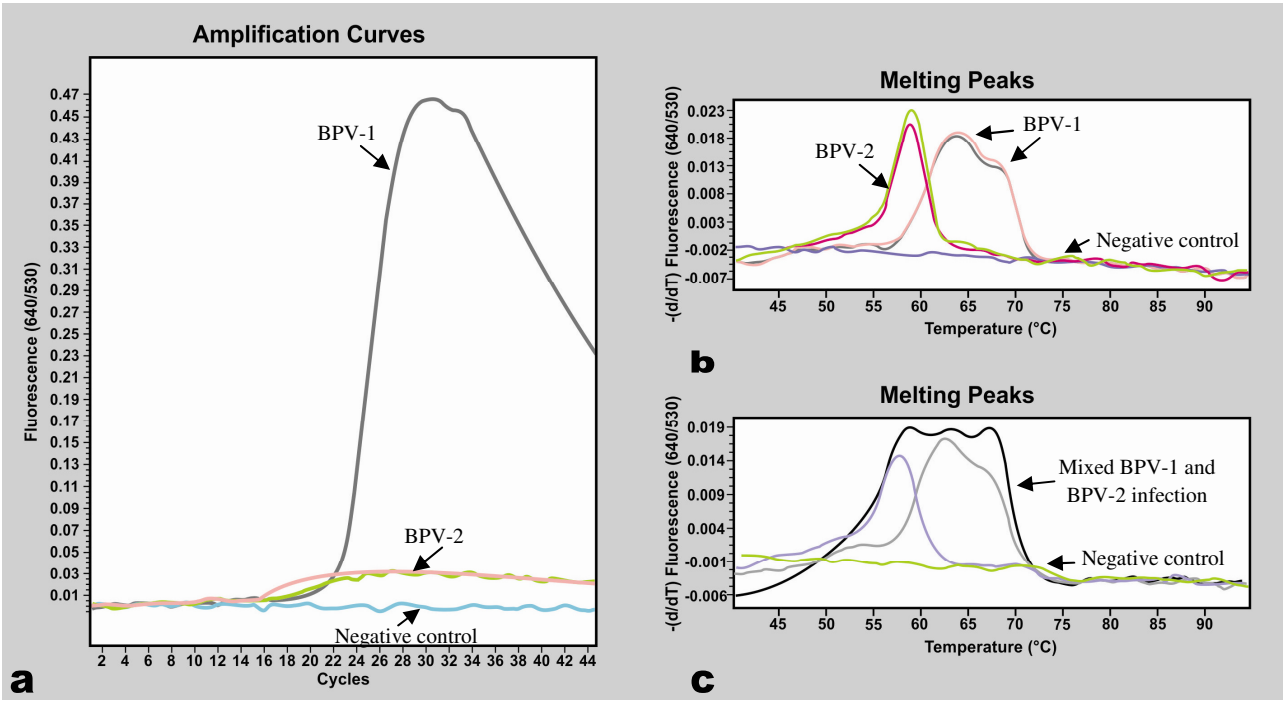

Figure 6 Real-time PCR results for the detection of BPV-1 DNA. (a) Amplification curves showing the increase in fluorescence at 640 nm. (b) Melting curve analysis of the amplicons generated from BPV-1 and BPV-2 positive DNA. Two peaks were obtained for BPV-1 DNA, at 62.90 ±1.24 °C and 68.17 ±0.71 °C. 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.

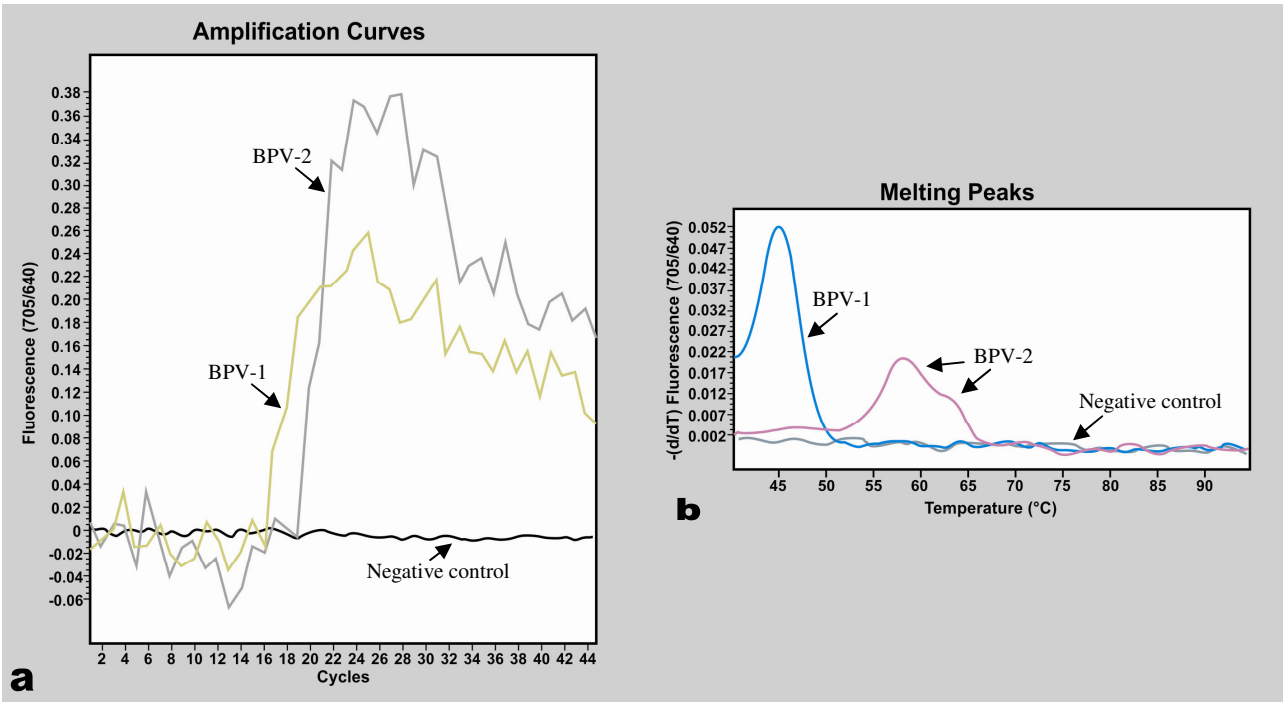

Figure 7 Real-time PCR results for the detection of BPV-2 DNA. (a) Amplification curves showing the increase in fluorescence at 705 nm. (b) Melting curve analysis of BPV amplicons. Two peaks were obtained for BPV-2 DNA, at 58.86 ±0.60 °C and 64.06 ±0.59 °C. The melting peak for BPV-1 DNA was at 46.25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

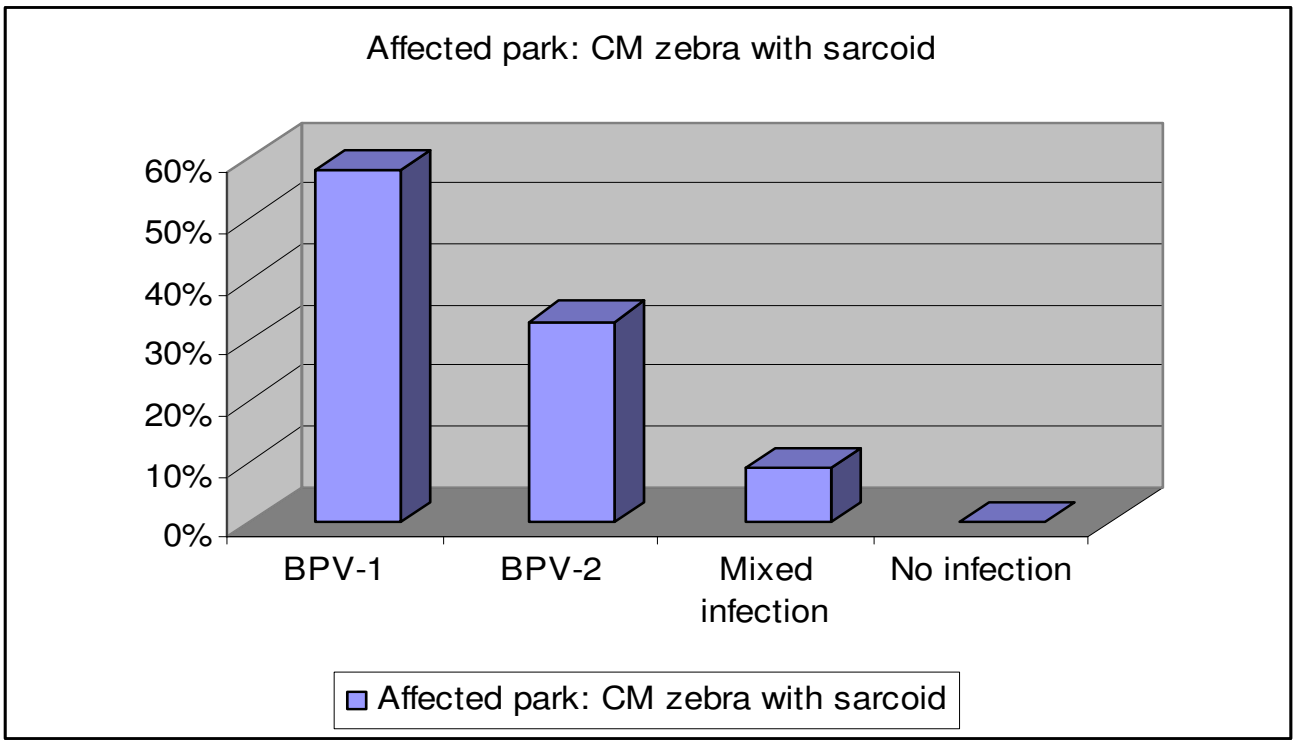

Figure 8 Prevalence of BPV infection in zebra sarcoid tumour samples collected from the Gariep Dam Nature Reserve, Bontebok National Park and Mountain Zebra National Park as determined with real-time PCR.

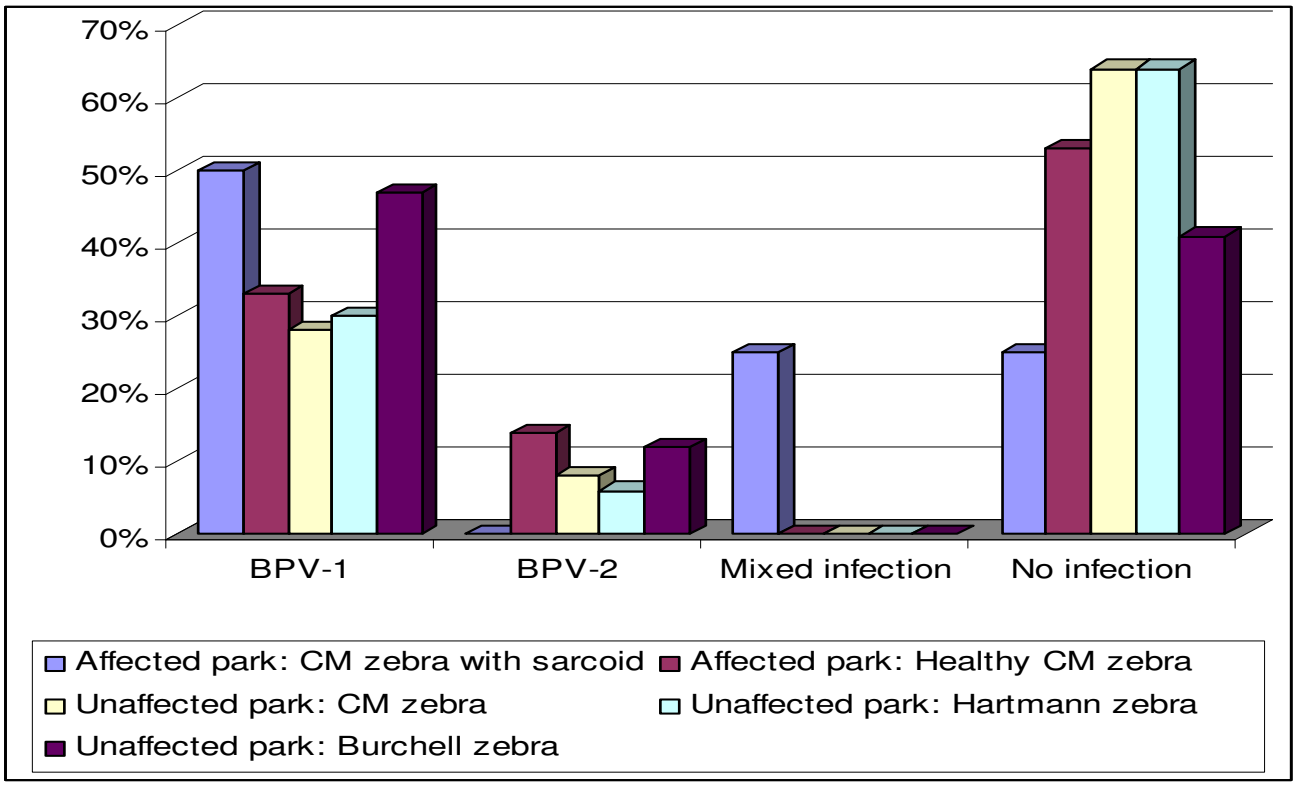

Figure 9 Prevalence of BPV infection in zebra blood collected from the various parks in South Africa as determined with real-time PCR. Affected park = where sarcoid has been observed; Unaffected park = where no sarcoid has been observed before; CM = Cape mountain zebra.

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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