

## Detection and characterisation of papillomavirus in skin lesions of giraffe and sable antelope in South Africa

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### ABSTRACT

Papillomavirus was detected electron microscopically in cutaneous fibropapillomas of a giraffe (*Giraffa camelopardalis*) and a sable antelope (*Hippotragus niger*). The virus particles measured 45 nm in diameter. Histopathologically, the lesions showed histopathological features similar to those of equine sarcoid as well as positive immunoperoxidase-staining of tissue sections for papillomavirus antigen. Polymerase chain reaction (PCR) detected bovine papillomavirus (BPV) DNA. Bovine papillomavirus-1 was characterised by real-time PCR in the sable and giraffe, and cloning and sequencing of the PCR product revealed a similarity to BPV-1. As in the 1st giraffe, the lesions from a 2nd giraffe revealed locally malignant pleomorphism, possibly indicating the lesional end-point of papilloma infection. Neither virus particles nor positively staining papillomavirus antigen could be demonstrated in the 2nd giraffe but papillomavirus DNA was detected by real-time PCR which corresponded with BPV-1 and BPV-2.

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

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buffalo (*Bubalis bubalis*)<sup>28</sup> and bison (*Bison bonasus*)<sup>21</sup>.

Earlier it was thought that apart from BPV 1 and 2, papillomaviruses are strictly species-specific and only the natural host is infected. Even under experimental conditions papillomaviruses did not appear to infect any host other than the natural one. Lesions usually attributed to papillomavirus infection have been most extensively studied in cattle<sup>2,9,14</sup> and horses<sup>3,7,11,24,25</sup>, but at least 50 mammalian species have been confirmed as being infected by species-specific papillomaviruses<sup>33</sup>. Papillomaviruses appear to be widespread and have been found in a large number of vertebrate species and are assumed to have co-evolved with their hosts<sup>4,5</sup>. Virtually all mammalian species are hosts for one or more papillomaviruses<sup>33</sup>.

### CASE HISTORIES

#### Giraffes 1 and 2

An adult female giraffe (*Giraffa camelopardalis*) (Giraffe 1) with extensive lesions in the skin of the dorsal neck and back was observed near Shingwedzi rest camp in the northern part of the Kruger National Park (KNP), South Africa (Fig. 1). She was in a group of 6 animals, 1 of which was a bull. The other animals in the group showed no lesions. This area is a natural habitat for giraffe as they occur in a variety of dry savannas ranging from scrub to woodland<sup>29</sup>. Approximately 1 year later a 2nd affected giraffe (Giraffe 2), was observed in the vicinity of Skukuza rest camp and the Kruger Gate in the southern part of the KNP (Fig. 2). This was also an adult female and part of a group of 5 of mixed sex and age. She was the only animal in the group exhibiting wart-like lesions that were especially prominent in the skin of her head and neck, but had also spread to the rest of her body.

#### Sable

A group of 30 sable antelope (*Hippotragus niger*) was kept as a breeding herd on a game farm in the Kimberley district, Northern Cape Province, South Africa.

### INTRODUCTION

Papillomaviruses are classified in the family *Papillomaviridae*<sup>6</sup>. This large family of animal and human viruses generally infects epithelial cells causing hyperproliferative lesions. Papillomavirus also has oncogenic potential<sup>18</sup>, infecting cutaneous and mucous epithelia in a variety of hosts through cuts or abrasions and induces the formation of papillomas or warts by targeting the keratinocyte, the viral growth accompanying the steady maturation of the cell to the surface<sup>31</sup>. These

tumours are generally benign and self-limiting and spontaneously regress with the animal recovering completely, but occasionally benign tumours may persist and become malignant by progressing to squamous cell carcinomas<sup>10</sup>.

Bovine papillomavirus induces exophytic papillomas of cutaneous or mucosal epithelia in cattle. Papillomas are benign tumours which generally regress uneventfully; however, they do occasionally persist and provide the focus for malignant transformation to squamous cell carcinomas, particularly in the presence of environmental co-factors. This has been experimentally demonstrated in cancer of the urinary bladder and upper alimentary tract in cattle feeding on bracken fern (*Pteridium aquilinum*)<sup>39</sup>. In equines BPV causes a locally invasive, fibroblastic skin tumour of horses, donkeys, mules<sup>23</sup> and zebras<sup>37</sup>. These tumours appear as different clinical entities and can be classified into 6 clinical types<sup>16</sup>. This cross-species infection of Equidae by BPV 1 and 2 is the only record until recently of a papillomavirus crossing the species barrier, the resulting tumour being known as a sarcoid<sup>22</sup>. Bovine papillomavirus has been demonstrated in lesions in the water

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Fig. 1: Multiple papillomas in the skin of Giraffe 1 euthanased in the vicinity of Shingwedzi rest camp. (Photograph reproduced with kind permission of D S van Dyk, SANPARKS.)



Fig. 2: Giraffe 2 euthanased in the vicinity of Skukuza rest camp. Note the presence of multiple nodular fibropapilloma lesions in the skin of her neck, head and ears.

This arid area of South Africa is not the natural habitat of sable as they are a savanna woodland species, but such game species are sometimes translocated to private land and reserves outside their normal range. One cow was lame in the right hind leg and a wart-like lesion of 60 × 60 mm was present in the skin of the lateral aspect of the distal part of the 2nd phalanx proximal to the right hind hoof. After surgical removal, growth recurred at the original site and other well-defined wart-like lesions were also noticed in the skin of the right shoulder and lip.

The purpose of this study was to determine electron microscopically if BPV was present in the lesions of these animals and, if so, to detect and distinguish between BPV-1 and BPV-2 DNA using real-time PCR<sup>37</sup>.

## MATERIALS AND METHODS

### Sample collection

Giraffe 1 was shot in May 2007 in the vicinity of the Shingwedzi rest camp (23°97'42"S, 31°42'58"E). Samples of the

lesions were collected for PCR by excising pieces of tissue 10 × 3 × 3 mm in size. These were stored in sterile tubes at 4 °C until analysis. Lesion samples for histopathological examination were stored in duplicate in 10 % neutral buffered formalin. The 2nd giraffe (Giraffe 2) was shot near Skukuza rest camp (24°59'16"S, 31°34'32"E) in October 2008 and samples were collected as described above. The sable antelope was anaesthetised and the lesion on the pastern surgically removed in an effort to relieve the lameness. Samples of the lesion were taken as described above.

### Electron microscopy

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

An unfixed skin sample of the sable cow was prepared for TEM by grinding the

tissue in a mortar with a pestle in a small volume of sterile water and the mixture was centrifuged at 13 000 rpm for 45 min. The resultant pellet was re-suspended in water, stained with 3 % phosphotungstic acid and a drop of the suspension placed onto a formvar- and carbon-coated grid for examination. A relevant area was also retrieved from the histological wax block (see below) of the skin sample from the pastern and was treated with 1 % OsO<sub>4</sub> in xylene and embedded in an epoxy resin.

### Histopathology

The formalin-fixed samples from both giraffes and the sable were dehydrated and paraffin wax-embedded for routine histological processing. All tumour sections were stained with haematoxylin and eosin for light microscopy, as well as immunohistochemically for evaluation of papillomavirus, using a polyclonal rabbit anti-papillomavirus antibody against chemically disrupted BPV-1, by the avidin-biotin technique<sup>13</sup>. A section of a bovine fibropapilloma was used as a positive control.

### DNA extraction

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

### Real-time PCR

The hybridisation probe real-time PCR assay, as described by van Dyk *et al.*, (2009)<sup>37</sup> was used for the detection and differentiation of BPV-1 and BPV-2 DNA in the giraffe (1 & 2) and sable lesions. The primers (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') amplified a 244 and 247 bp region of the E5 ORF of BPV-1 and BPV-2 respectively. For the specific detection of BPV-1 amplicons, hybridisation probes BPV-1 anchor (5'-ACT GG TGT ACT ATG CCA AAT CTA TGG TTT CTA TTG-Fluor-3') and BPV-1 sensor (5'-LC-640-CTT GGG ACT AGT TGC TGC AAT GCA ACT-Pho-3') were used, and for the detection of BPV-2 amplicons, hybridisation 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 used.

All primers and fluorescent-labelled hybridisation probes were manufactured by Metabion International AG (Martinsried, Germany).



The PCR amplification mixture and reaction conditions were as previously described<sup>37</sup>. Plasmids obtained from Giraffe 1 and the sable were used as template DNA (2  $\mu$ l) and genomic DNA from Giraffe 2 was used. Fluorescence was measured at 640 and 705 nm and the results were analysed with the Roche LightCycler<sup>®</sup> Software v4.0. Specimens, from positive zebra sarcoid (BPV-1 and BPV-2)<sup>37</sup> were included as positive controls.

#### Cloning and sequence analysis

Conventional PCR was used for the amplification of a 637 bp DNA fragment of a region of the E5 ORF of both BPV-1 and BPV-2. The primers (F\_3610: 5'-GCT AAC CAG GTA AAG TGC TAT C-3'; R\_4247: 5'-TGC TTG CAT GTC CTG TAC AGG T-3'), PCR amplification mixture and reaction conditions were as previously described<sup>37</sup>. However, a nested PCR step was included using 1  $\mu$ l of the 1st round amplicons as PCR template in Giraffe 1 and the sable. The amplicons obtained were purified, cloned into the pGEM<sup>®</sup>-T easy vector (Promega pGEM-T Easy Vector System, Promega, Madison, USA), and transformed into competent JM 109 *E. coli* cells (Promega, Madison, USA). Recombinant plasmid DNA was isolated, directly sequenced using the ABI BigDye<sup>™</sup> Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) and analysed on an ABI 3100 sequencer at Inqaba Biotec (Pretoria, South Africa). Sequencing data were assembled and edited with the GAP4 programme of the Staden package (version 1.6.0 for Windows)<sup>30</sup> and aligned with published sequences of related genera using ClustalX (version 1.81 for Windows). A BLAST search was performed using the Blastn algorithm. Phylogenetic trees were constructed using the maximum parsimony method (Mega 3.0 software package)<sup>17</sup> in combination with the bootstrap method<sup>12</sup> (1000 replicates/tree for distance methods and 100 replicates/tree for parsimony methods). Human papillomavirus type 16 (K02718) was used as an outgroup.

## RESULTS

### Macroscopic lesions

There were extensive skin lesions on the head, neck and back of the 2 adult female giraffes (Giraffe 1 and 2) in the Kruger National Park (Figs 1, 2).

### Electron microscopy

Negatively-stained spherical papillomavirus particles with a diameter of 52 nm and distinct capsomeres were present in the unfixed skin sample of the

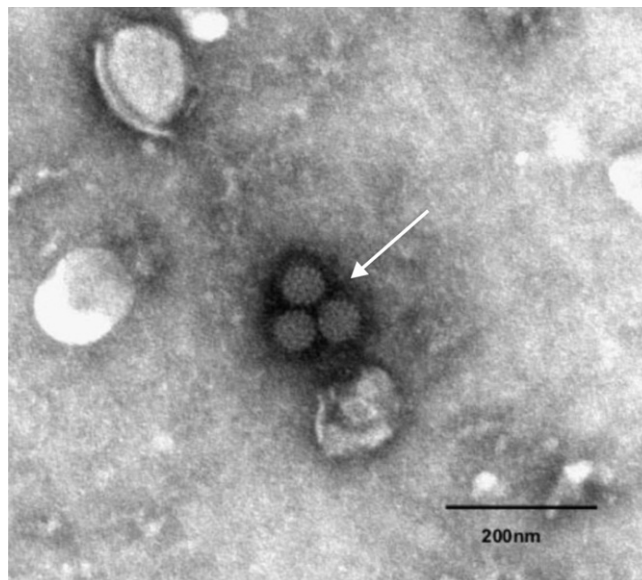


Fig. 3: Negatively-stained intranuclear papillomavirus particles in an unfixed sample from the sable cow.

sable cow (Fig. 3). The nuclei of the *stratum granulosum* of both the sable cow and Giraffe 1 contained numerous randomly scattered papillomavirus particles measuring 45 nm in diameter (Figs 4, 5). The nuclei of infected cells displayed irregular clumps of condensed chromatin. No virus was demonstrated in samples obtained from Giraffe 2.

### Histopathology

The skin lesions of Giraffe 1 were fibropapillomatous in nature and those of the sable resembled equine verrucous sarcoid. Small nuclei in the *stratum granulosum* of both animals stained immunohistochemically positive for papillomavirus antigen. The lesions of Giraffe 2 were more sarcoid-like, expansile and infiltrative in the dermis. Lesions of both giraffes showed evidence of malignancy. Scattered, large fibroblasts with

bizarre large nuclei, some of which appeared multinucleated, occurred throughout the dermal tumour tissue with those of Giraffe 2 being more prevalent. Immunohistochemical staining of sections from Giraffe 2 did not demonstrate papillomavirus antigen. The detailed histopathology is described and illustrated elsewhere<sup>38</sup>.

### Real-time PCR

The primers used in the real-time PCR assay<sup>37</sup> amplified either a 244 bp or 247 bp region of the E5 ORF of BPV-1 and BPV-2, respectively. Two separate hybridisation probes sets were used in a multiplex format for the specific detection of BPV-1 and BPV-2 DNA. For the detection of BPV-1 DNA, an increase in fluorescence is expected at 640 nm as well as 2 BPV-1-specific melting peaks at  $62.90 \pm 1.24$  °C and  $68.17 \pm 0.71$  °C. Similarly, for

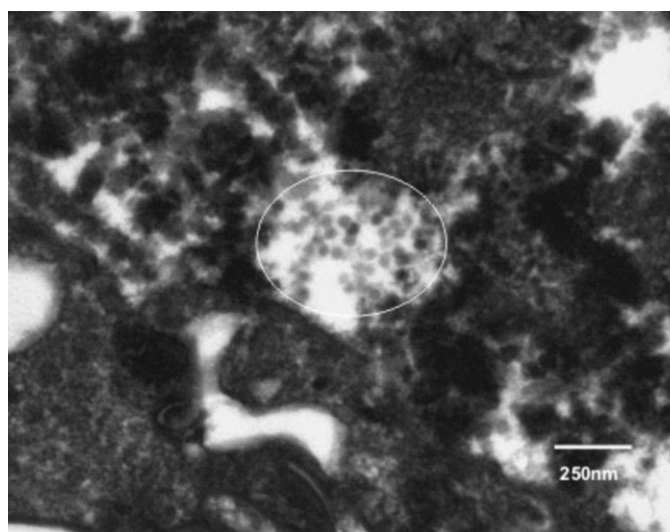


Fig. 4: Intranuclear papillomavirus (encircled) particles stained positively with uranyl-acetate in ultra-thin sections of wax-retrieved epoxy sections of the sable cow skin.

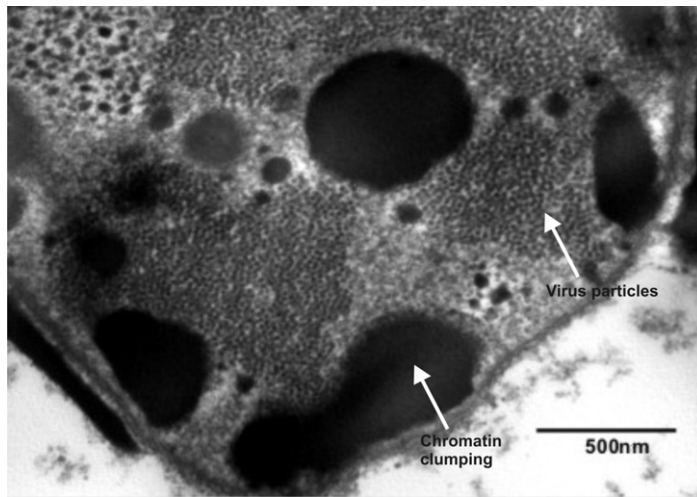


Fig. 5: Numerous intranuclear papillomavirus particles in sections of formalin-fixed skin of Giraffe 1. Note nuclear chromatin clumping.

the detection of BPV-2 DNA, an increase in fluorescence at 705 nm is expected as well as 2 BPV-2-specific peaks at  $58.86 \pm 0.60$  °C and  $64.06 \pm 0.59$  °C.

Bovine PV-1-positive material from sarcoids of Cape mountain zebras induced a graph with melting peaks showing a similar configuration to those of material from Giraffe 1 and the sable. Giraffe 2 demonstrated fluorescence at 58 °C as well as 68 °C at 640 nm (Fig. 6) indicating both BPV-1 and BPV-2.

#### Cloning and sequencing

Fragments, 636 bp in size, of the E5 ORF of the sable and Giraffe 1 samples were amplified, cloned and sequenced to confirm that the amplicons obtained were indeed related to BPV. Sequences were edited and truncated to a length of 478 bp. Six [K10 (HM446170); K1 (HM446171); K3 (HM446172); K7 (HM446173); K5 (HM446174); K1-1 (HM446174)] of the 9 Giraffe 1 clones had identical sequences over the 478 bp region and a BLAST search revealed that these sequences were identical to those of the BPV -1 E2 and E5 protein encoding genes previously reported<sup>37</sup> to occur in Cape mountain zebras (FJ648519 to FJ648528). The other 3 clones [K4 (HM446168); K27 (HM446169); K2 (HM446176)] were not identical to each other, but the BLAST search showed the highest similarity (~99 %) with the BPV type 1 E2 and E5 protein-encoding genes found in Cape mountain zebras (FJ648519 to FJ648528). Only 2 sequences were obtained from sable clones, Sable 4 (HM 446166) and Sable 2 (HM 44167) and they showed 2 nucleotide differences within the 478 bp fragment. Again, the BLAST search showed the highest similarity (~99 %) with the BPV- E2 and E5 protein encoding genes found in Cape mountain zebras. All sequences obtained from Giraffe 1 and

sable samples were closely related (~97 %) to the BPV-1 subtype IV E5 protein-encoding gene (accession number AY232260) detected in equine sarcoid<sup>11</sup>. A phylogenetic tree was constructed and the results were concurrent with the BLAST results obtained (Fig. 7). In the case of Giraffe 2, no good-quality sequence data could be obtained and the DNA could only be detected by real-time PCR.

#### DISCUSSION

Numerous descriptions of papillomatous epithelial growths in the skin of many species of mammals have been reported<sup>15,21,26–28,32,33,36</sup>. Epithelial growths were reported in giraffes in the Kruger National Park in 1978<sup>35</sup>. They are characterised variably by a conspicuous amount of fibrous connective tissue underlying hyperplastic epithelium and are commonly associated with infection with a papillomavirus. This study documents the presence of BPV and/or DNA in such growths in the skin of the 2 giraffes and a sable antelope.

Papillomatosis is a naturally-occurring, generally species-specific infectious disease. The lesions are regarded as hyperplastic or a form of benign neoplasia as they do not metastasise internally<sup>20</sup>. Although bovine-associated papillomavirus DNA is consistently found in the sarcoid lesions of the horse<sup>1,19</sup> and zebra<sup>37</sup>, papillomavirus particles have not been demonstrated and the disease is therefore considered to be a non-productive infection in which the viral DNA exists episodically in these species. Most fibropapillomas (sarcoids) in horses contain identifiable BPV DNA of either type 1 or 2<sup>3,7,11,24,25,34,40</sup>. The E1 gene of the papillomavirus identified in sarcoids of donkeys is very similar to BPV-1 and it has been proposed that it is a subtype of BPV-1<sup>25</sup>. In the

present study the papillomavirus particles that were demonstrated in the wart-like lesions of the sable antelope and Giraffe 1 are considered to be closely related to BPV-1.

Ultrastructurally, virus particles have also been demonstrated in skin papillomas in an impala and a giraffe in Kenya<sup>15</sup> but sequencing was not done. In the water buffalo<sup>28</sup> and the bison<sup>21</sup> virus has also been demonstrated electron microscopically, and following its sequencing it was reported to be homologous to the LCR of BPV-1 (prototype sequence, accession number X02346) in the water buffalo<sup>28</sup> while the DNA sequence of 413 bp amplicon derived from the European bison compared with the consensus sequence of BPV-2 (GenBank Accession number AY300818)<sup>21</sup>. The virus was also demonstrated in both species of animals by electron microscopy<sup>21,28</sup>.

The real-time PCR method<sup>37</sup> demonstrated the presence of BPV-1 in the sable and Giraffe 1 and was confirmed by cloning and sequencing. In the 2nd giraffe, no good-quality sequences could be obtained, although, real-time assay demonstrated the presence of both BPV-1 and BPV-2. This can be attributed to the high sensitivity of the real-time PCR assay used, which is capable of demonstrating the virus at less than 1.5 gene copies<sup>37</sup>. Another possible reason why the virus could not be demonstrated was that the papilloma had undergone a degree of cellular malignant transformation, with more pleomorphic fibroblasts being present in the lesions than in Giraffe 1. The structural integrity of the virus appeared to be lost and the virus or viral antigen was no longer present<sup>20</sup>.

Although the geographical areas are widely separated, the Giraffe 1 and sable clones clustered together with high similarity to BPV-1 sequences, and with some of the zebra sequences reported previously<sup>37</sup>. None of the sable or Giraffe 1 sequences reported here were of the BPV-2 group, and only some of the previously reported zebra samples<sup>37</sup> clustered in BPV-2. The human papillomavirus type 16 outgroup clustered completely differently (Fig. 7).

Clinical manifestation of latent infection in the 3 animals described here may have been provoked by stress. In the case of the sable antelope, not being in its typical habitat may have induced immunosuppression. In the case of the giraffes it can only be speculated that drought conditions and the resultant high tannin content of the *Acacia* trees as a result of over-browsing acted as a co-factor in BPV-associated carcinogenesis as seen in cattle following bracken fern ingestion<sup>8</sup>.

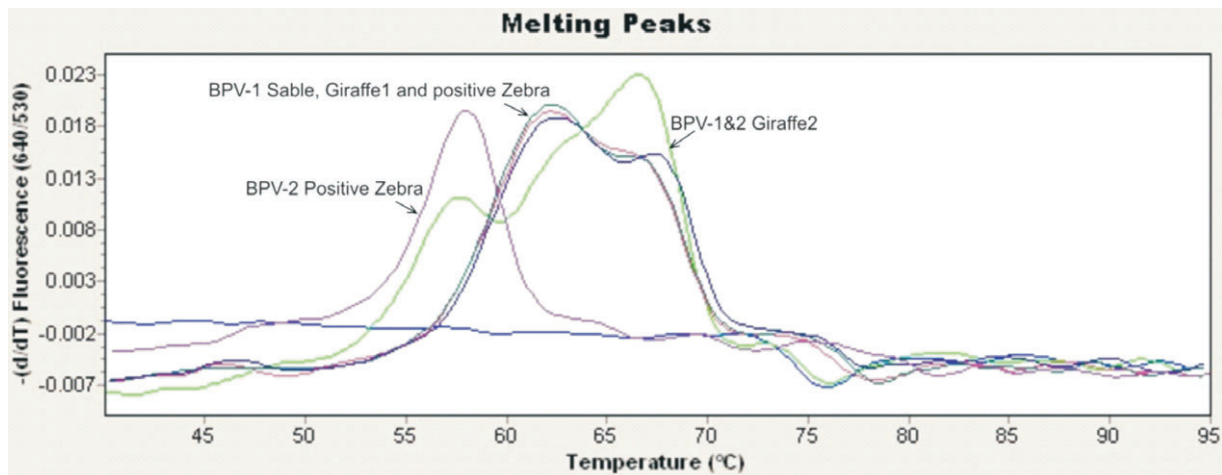


Fig. 6: Real-time PCR results of the sable and Giraffe 1 demonstrating BPV-1 DNA, and showing an increase in fluorescence at  $62.90 \pm 1.24$  °C in both. The positive BPV-1 control zebra shows the same pattern while the control zebra for BPV 2 reveals an increased fluorescence at  $58.48 \pm 0.61$  °C. Giraffe 2 demonstrates a different graph fluorescing at 58 °C as well as 68 °C.

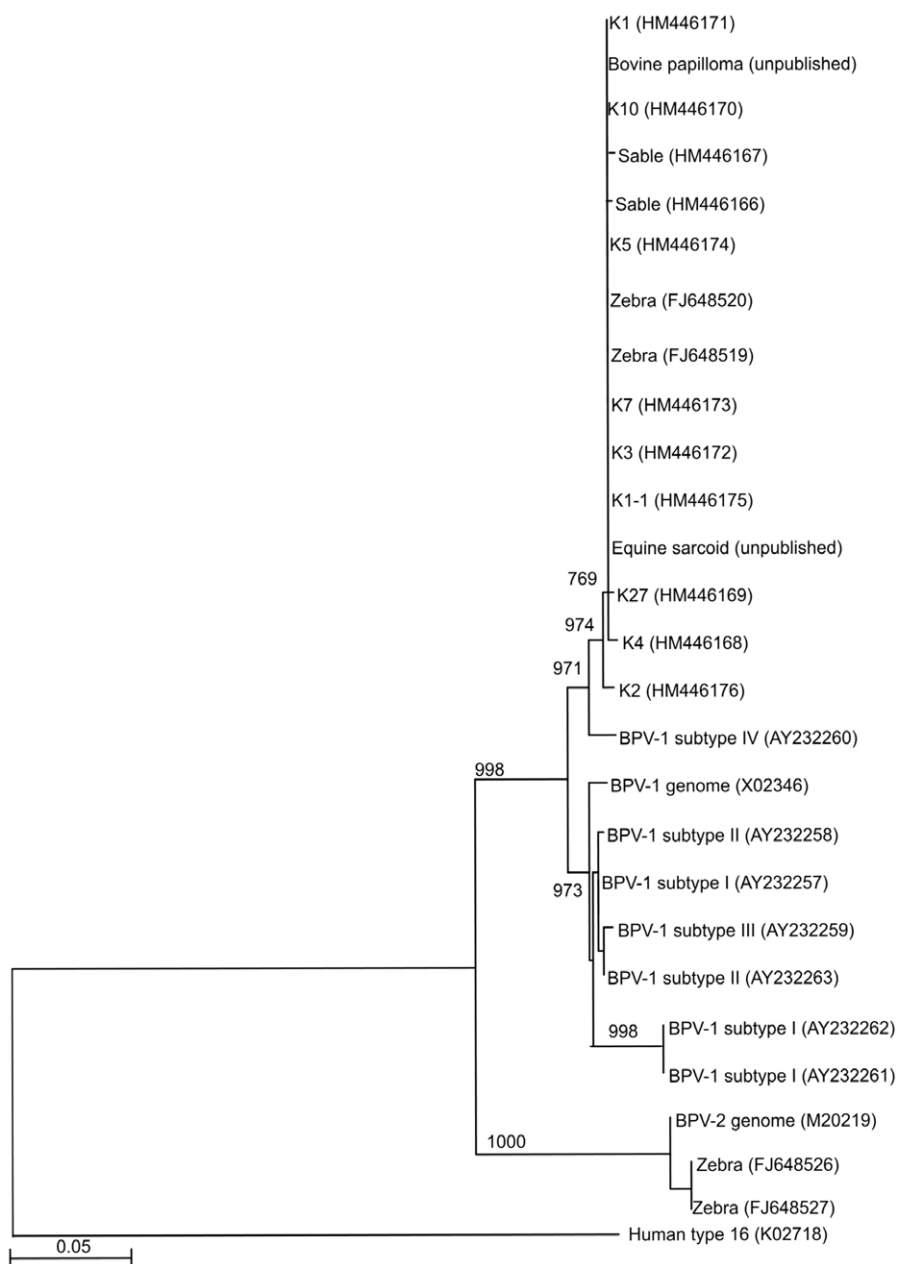


Fig. 7: Results of the tree of BPV E2E5-encoding gene showing the phylogenetic relationship compared with sequences of BPV-1 and BPV-2 obtained from GenBank.

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