



OPEN Accurate identification of bovine deltapapillomavirus in equine sarcoids by ddPCR

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Sarcoids are benign and locally aggressive skin lesions that commonly affect horses and other equid species. Sarcoids are generally considered to be caused by bovine delta-papillomaviruses (δPVs) types 1 and 2 (BPV1 and BPV2, respectively). Moreover, while bovine δPV types 13 and 14 (BPV13 and BPV14, respectively) are also suspected to induce sarcoids, information regarding this possibility and the occurrence of multiple bovine δPV infections in sarcoids is scarce. This study aimed, for the first time, to assess BPV1, BPV2, BPV13, and BPV14 infections and co-infections in equine sarcoid samples of Austrian provenance, and to determine the intralesional DNA loads of the detected bovine δPV types using highly sensitive droplet digital polymerase chain reaction (ddPCR). BPV DNA was detected in 93 sarcoid samples. The analyses revealed that BPV1 was the predominant bovine δPV type in sarcoids from Austria, with 83/93 lesions testing BPV1-positive. Importantly, 66 tumors also contained BPV2 DNA. In six cases, a triple infection including BPV13 or BPV14 was noted, and one lesion showed a quadruple infection. This is the first ddPCR-based study to show multiple infections by all four bovine δPVs in equine sarcoids. Clinical data suggest that BPV1/2 co-infection may be associated with more severe and therapy-resistant disease. In-depth studies are required to investigate this possibility in greater detail.

Keywords Bovine papillomavirus, Droplet digital PCR (ddPCR), Co-infection, Horse, Sarcoid

Sarcoids are the most common tumors in equids, affecting up to 12% of horses worldwide¹. Sarcoids are skin neoplasms that do not metastasize. Based on their clinical appearance, sarcoids are classified as mildly occult, verrucous, nodular, fibroblastic, mixed, or malignant lesions². Importantly, sarcoids are often resistant to therapy and tend to reoccur in more extensive and multiple forms following ineffective treatment. Consequently, sarcoid disease constitutes a serious welfare problem and negatively affects the horse industry³.

Sarcoids are generally believed to be caused by bovine papillomavirus (BPV) types 1 and 2 (BPV1 and BPV2, respectively), and are probably associated with accidental or iatrogenic trauma^{1,3}. In addition, a growing body of evidence indicates that BPV type 13 (BPV13), and possibly type 14 (BPV14), can induce sarcoid lesions in equids^{4–6}.

Papillomaviruses (PVs) are a family of small DNA viruses that cause benign skin lesions and malignant tumors in vertebrates^{7,8}. Typically, PVs are strictly epitheliotropic and highly species-specific. The small genus of delta-papillomaviruses (δPVs) is an exception to this rule⁹. The members of this genus, including bovine δPVs 1, 2, 13, and 14 (BPV1, BPV2, BPV13, and BPV14, respectively) can also infect fibroblasts. This particular feature may explain their wide host range, which includes horses and other equid species³.

Sarcoid formation is primarily induced by BPV1 and/or BPV2. However, the prevalence and distribution of these two genotypes varies geographically. In Western United States of America (USA) and Oceania, sarcoids predominantly harbor BPV2^{10,11}. In Eastern USA and Europe, most sarcoids are induced by BPV1, and BPV2 is rarely detected^{3,10,12}. In some cases, sarcoids contain both BPV types, yet almost no information on bovine δPV co-infections in sarcoids has been reported so far¹³.

BPV13 was first identified and characterized in a cow cutaneous papilloma in Brazil. This virus is also involved in the pathogenesis of bladder tumors in European cattle¹⁴ as well as squamous and fibropapillomas in cattle rumen in Brazil¹⁵. Although BPV13 DNA was detected in sarcoids affecting horses in Brazil^{4,5}, no BPV13 DNA has been detected in sarcoids affecting European horses so far¹⁶.

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GENOTYPES	OVERALL POSITIVE SAMPLES	%	SINGLE INFECTION	%	MULTIPLE INFECTION	%
BPV1	83	89.3	22	73.3	61	75.2
BPV2	66	71.0	8	26.7	58	90.5
BPV13	5	5.4	N.D	-	5	6.8
BPV14	10	10.8	N.D	-	10	13.6

Table 1. Overall bovine δ PV DNA prevalence in sarcoid samples, and in single and multiple infections is shown. N.D. = Not detectable.

Coinfections	Genotype combinations	Number
Double	BPV1/2	49
	BPV1/13	2
	BPV1/14	3
	BPV2/13	1
	BPV2/14	1
Triple	BPV1/2/13	1
	BPV1/2/14	5
Quadruple	BPV1/2/13/14	1

Table 2. Genotype combinations in multiple BPV infections.

Little is known regarding the occurrence and geographical distribution of BPV14. Genomic BPV14 sequences have been detected in felid skin lesions in the USA and Oceania^{17–19}, bovine papillomas and inflammatory skin conditions in Australia²⁰, and urinary bladder tumors in Italy⁶. To date, no reports have described the detection of BPV14 in equine sarcoids. However, the almost identical genomic organizations of BPV1, BPV2, BPV13, and BPV14, and the high similarities among these PVs at the DNA and protein levels suggest that BPV14 can potentially infect and transform equine fibroblasts¹⁹.

Digital polymerase chain reaction (dPCR) is a new-generation traditional quantitative PCR²¹. As such, droplet dPCR (ddPCR) provides more precise and reproducible detection of pathogen load in the clinical diagnosis of infectious diseases, including viral diseases²². ddPCR has been shown to be a very sensitive diagnostic tool for the detection and quantification of human PV (HPV) DNA^{23–25}, BPV DNA in cattle²⁶, as well as ovine (OaPV) and caprine PV (ChPV) DNA in small ruminants^{27,28}. Given that ddPCR was previously shown to outperform qPCR in terms of sensitivity and yield significantly fewer false results^{23,26–28}, we have used this method for the first time to assess BPV1, BPV2, BPV13, and BPV14 infections and co-infections in equine sarcoid samples of Austrian provenance, and to determine the intralesional DNA loads of the detected bovine δ PV types.

Results

ddPCR detected and quantified BPV DNA in 93 of the 96 (~96.9%) equine sarcoid samples. BPV DNA was not detected in three (~3.1%) sarcoid samples. ddPCR detected BPV1, BPV2, BPV13, and BPV14 DNA in 83 (~89.3%), 66 (~71%), five (~5.4%), and 10 (~10.8%) samples, respectively (Table 1). Single infections were detected in 30 samples (~32.3%), and multiple infections were identified in 63 samples (~67.7%). No single infections caused by BPV13 or BPV14 were detected. BPV1 and BPV2 were responsible for 22 (~73.3%) and eight (~26.7%) single infections, respectively (Table 1).

Double infections were the most prevalent multiple infections and were detected in 56 patients (~89%). The BPV1/BPV2 genotype combination was observed in 49 samples (~87.5%). The remaining seven double infections (~12.5%) were characterized by the BPV1/13 and BPV1/14 genotype combinations in two and three samples, respectively, and the BPV2/13 and BPV2/14 genotype combinations in one sample each. ddPCR detected triple infections in six samples (~9.5%), five of which showed the BPV1/2/14 genotype combination, and one showed the BPV1/2/13 genotype combination. A quadruple infection was observed in only one sample (Table 2).

The Mann–Whitney test showed no significant differences in viral load between single and multiple infections. BPV1 showed the highest number of copies/ μ L. Seventy-one sarcoid samples showed a BPV1 DNA copy number varying from 259 to 20,585 copies/ μ L. Only 12 samples contained fewer than 259 BPV1 DNA copies/ μ L. BPV2 was detected and quantified at lower concentrations. Only isolated samples showed very high BPV2 DNA copy numbers (up to 19800 copies/ μ L). BPV13 and BPV14 DNA were quantified at low copy numbers, with the highest copy numbers for these genotypes being 7.7 and 8.2 copies/ μ L, respectively. BPV1 showed significant differences in viral load in comparison with the remaining BPV genotypes using the Friedman test ($P < 0.05$). Indeed, after adjusting for the Bonferroni multiple-comparison correction, the P value was < 0.001 . Finally, Fig. 1 shows the ddPCR rain plots for all delta-papillomaviruses. Supplemental Table S1 reports the raw data from all the 96 samples in which BPV infection was identified using ddPCR.

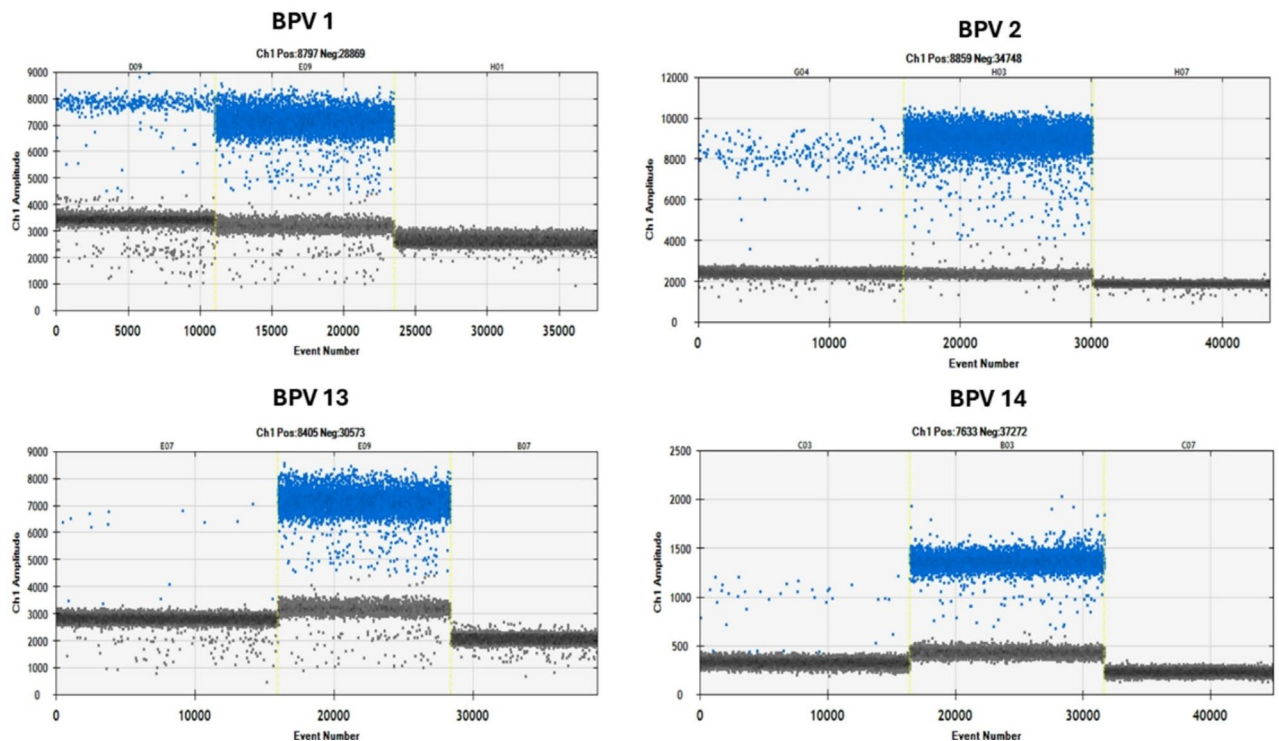


Fig. 1. Equine sarcoid. QuantaSoft screenshots show the rain plots of the ddPCR results related to BPV genotypes. Positive plots are represented in blue, whereas negative droplets are in grey. BPV1–D09: positive samples; E09: is the positive control; H01: negative samples. BPV2–G04: positive samples; H03: is the positive control; H07: negative samples. BPV13–E07: positive samples; E09: is the positive control; B07: negative samples. BPV14–C03: positive samples; B03: is the positive control; C07: negative samples.

Discussion

Sarcoids are the most common tumor disease in horses and other equid species and have a major influence on the health and welfare of the affected animals. Although the causal association between BPV1/2 infection and sarcoid onset and progression is well established³, many aspects of this disease remain poorly understood. The unclear issues include (i) the prevalence of BPV13 and BPV14 in equine sarcoids, (ii) the incidence of co-infections by bovine δ PVs in the lesions, and (iii) the possible pathobiological significance of bovine δ PV co-infections in sarcoid disease. To address these gaps in knowledge, we tested tumor DNA isolates with confirmed PCR-compatible quality for the presence of bovine δ PV DNA by using ddPCR technology.

As expected, consistent with data previously obtained in Austria^{16,29,30}, the majority of sarcoid DNA isolates harbored BPV1. In 32.3% of the lesions, BPV1 represented the only detected PV type, whereas approximately 87.5% of tumor samples showing double infections contained the BPV1 and BPV2 genotype combination. The latter finding was surprising, since a single BPV2 infection was detected in only eight cases. Triple and quadruple infections were observed much less frequently and consistently showed BPV1 and BPV2 in combination with BPV13 and/or BPV14.

To the best of our knowledge, this is the first study to evaluate both the detection and virus load of bovine δ PVs associated with equine sarcoids using ddPCR technology. Notably, ddPCR has been already shown to be an accurate, sensitive, and specific molecular assay for detection and quantification of bovine δ PVs in healthy and diseased cattle^{26,31}, sheep³², goats³³, and healthy horses³⁴ as well as of OaPV DNA in equine sarcoids³⁵ and in commercial semen of stallions³⁶.

Interestingly, some tumor DNA samples that had scored BPV1/2 PCR-negative in Vienna were subsequently tested using the ddPCR tool in Naples, and scored positive for BPV1 or showed a double BPV2/BPV14 co-infection. These findings agree with the concept that ddPCR outperforms other PCR-based approaches³⁷, and indicate that ddPCR can detect otherwise undetectable bovine δ PVs²⁵. Furthermore, ddPCR provided novel information about the presence of BPV13 and BPV14 DNA, co-infections, and type-specific viral DNA loads in the tested sarcoids, thereby providing a better understanding of geolocalization and territorial distribution of bovine δ PV genotypes.

To the best of our knowledge, this is the first study to provide evidence of BPV13 infection in sarcoid-affected horses from Austria and of BPV14 infections associated with neoplastic pathology in equids, thus confirming the findings of a recent study that included BPV13 and BPV14 in the equine biota³⁴. However, the low BPV13 and BPV14 DNA loads indicated the possibility that their detection was the result of contamination events. Nevertheless, the detection rates (approximately 6.8% and 13.6%, respectively) were consistent with the previous findings and supported the suggestion that our findings were attributable to actual viral infections. Furthermore,

the positive, negative, and no-template controls included in ddPCR yielded the expected results, indicating that the reaction was conducted accurately and corroborating our findings. Very little information is available on the epidemiology of BPV13 and BPV14 infections in horses, which could explain the poor understanding of the biological significance of cross-species transmission of these viruses. Therefore, more research is needed to define the role of BPV13 and BPV14 in the pathogenesis of equine sarcoids and to determine whether the clinical implications of sarcoids differ in relation to the causative BPVs.

In few BPV1/2-positive samples, ddPCR did not detect any BPV genotype. These samples were repeatedly subjected to virus PCR screening in Vienna; thus, the DNA was frozen and thawed several times prior to their transfer to Naples. Therefore, we assumed that the negative ddPCR results for these samples might be due to DNA degradation. Furthermore, we do not exclude the possibility that novel, unknown bovine δ PVs and/or involvement of other bovine PV types that are not classified within δ PV genus could have caused these neoplasms. It is conceivable that some equine sarcoids can be viral lesions characterized by a high genetic PV diversity, which may make current molecular tools enable to detect PV in some cases. Our suggestion appears to be corroborated by recent molecular studies based on PV amplification and sequencing^{38–40}.

Our molecular findings also characterized multiple infections. Multiple PV infections have potential oncogenic risks that remain unclear, and their clinical significance in comparison with those of single infections remains controversial. Some studies have indicated that co-infection enhances the risk of PV-related disease and poorer clinical outcomes^{41,42}; in contrast, other studies have shown that multiple infections have no synergistic or additive effects on the development of PV-related disease^{43–46}.

Determining the epidemiology of single infection and co-infections of bovine δ PVs may be important with the aim of identifying territorial genotype distribution and establishing appropriate prevention strategies, such as designing new pharmacological and/or immune therapeutic protocols that could be tailored to each animal species.

In this context, it is worthwhile remembering that bovine δ PV infection impairs immune response of the host that plays an important role in the pathogenesis of equine sarcoids. Bovine δ PVs develop mechanisms via their E5 oncoprotein that lead to the shutdown of the host immune system, which allows these viruses to escape immune response and facilitates persistent viral infection^{47,48}.

Methods

Ethics statement

This study was conducted in accordance with relevant guidelines and regulations. All methods are reported in accordance with ARRIVE guidelines (<https://arriveguidelines.org>).

The animal collection and handling and tissue sampling procedures were performed in accordance with the ethics guidelines of the Veterinary University Vienna and Austrian Law. Permission to collect material was obtained by oral informed consent from the animals' owners who were previously informed and in agreement with the purpose and methods used.

Sample collection

The 96 samples included in this study were obtained from 95 equids residing in Austria. They consisted of tissue aliquots, superficial swabs, or scrapings from lesions with diagnosis of equine sarcoids. The samples were collected between 2018 and 2024 during therapeutic surgical excision at the Centre of Equine Health and Research (CEHR) of the University of Veterinary Medicine in Vienna, Austria, or sent to the University's Research Group Oncology (RGO) by referral vets for diagnostic purposes. The horse and sample specifications are listed in Supplemental Table S2.

Standard PCR screening

DNA was extracted from all tissue samples using a DNeasy Blood & Tissue kit in accordance with the manufacturer's instructions (Qiagen, Hilden, Germany). Subsequently, the DNA isolates were tested using equine beta-actin PCR (EBA PCR), as described previously⁴⁹, to confirm their PCR compatibility. In short, primers 5'EBA and 3'EBA (5'-TCACCCACACTGTGCCCATCTACG-3' and 5'-CGTCRTACTCCTGCTTGCTGATCC-3'; made by Eurofins, Vienna, Austria) were selected from the sequence corresponding to GenBank accession no. AF035774 and used for amplification of a 614-bp sequence using Phusion™ Hot Start II DNA Polymerase (Thermo Fisher Scientific, Vienna, Austria) according to the manufacturer's instructions. The cycling program was performed in a Bioer LifeECO cycler (Biozym, Hessisch-Oldendorf, Germany) and consisted of 98 °C for 2 min followed by 40 amplification cycles (98 °C for 15 s, 69 °C for 30 s, 72 °C for 30 s) and a final elongation step at 72 °C for 5 min.

In the next step, all EBA-positive DNA isolates (100%) were screened for the presence of BPV1/2 E5 DNA using an established PCR protocol with primers 5'BPV1/2-E5 and 3'BPV1/2-E5 (5'-CACTACCTCCTGGAA TGAACATTTCC-3'; 5'-CTACCTTWGGTATCACATCTGGTGG-3'; Eurofins) selected from homologous regions up- and downstream of the E5 ORF of BPV1 and BPV2 (Genbank IDs X02346 and M20219.1). This system does not allow for distinction between BPV1 and BPV2⁵⁰. Confirmed BPV1-positive sarcoid DNA, non-infected equine skin DNA, and sterile water were included in each reaction as positive, negative, and no-template controls, respectively. The reactions were performed as described above. EBA and E5 PCR products (16 µL) were separated by gel electrophoresis on 1.5% Tris-acetic acid-ethylenediaminetetraacetic acid (TAE) gels and then visualized by ethidium bromide staining. In the final step, 50-µL aliquots of all EBA-positive DNA isolates were consecutively sent in two batches (batch #1: fall 2023, batch #2: spring 2024) to the Università degli Studi di Napoli Federico II for ddPCR-based analyses.

Forward 5' 3'		Reverse 5' 3'	Probe	region - size
BPV1	ACTTCTGATCACTGCCATT	ATAGAAACCATAGATTGGCA	TGAAGTGTTCTGTTTGTA-FAM	ORF E5 - 67 bp
BPV2	TACAGGTCTGCCCTTTAAT	AACAGTAAACAAATCAAATCCA	AACAACAAAGCCAGTAACC-FAM	3'UTR E5-77 bp
BPV13	CTGTGTGGATTGATTGTT	CAGGGGAATACAAATTCT	TGAAGTGTTCTGTTTGTA-FAM	3'UTR E5-98 bp
BPV14	CTTTGTTATTGTATATGAGTCTGT	ACTCTTGACGGTTTAAAAGTA	ATCTTGCCAGTGATCCTG-FAM	3'UTR E5 - 98 bp

Table 3. Primers and probes used for detection of the four BPV genotypes by ddPCR.

Droplet digital PCR

The samples obtained from Vienna were photometrically analyzed using a NanoVue Plus system (GE Healthcare, Boston, MA, USA) to determine their respective DNA concentrations. Then, aliquots were adjusted to concentrations of 100 ng/7 μ L. ddPCR was performed as described previously⁵¹ using the QX100 ddPCR System according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). Each reaction was performed in a final volume of 22 μ L, which consisted of 11 μ L of ddPCR 2x Supermix for Probes (Bio-Rad Laboratories, Hercules, CA, USA), 0.9 μ M of each primer, 0.25 μ M of probe, and 7 μ L of sample DNA (100 ng).

The ddPCR primers and probes for amplification of 67- to 98-bp sequences were selected within the E5 coding and non-coding regions according to the following sequences deposited in the GenBank: MH197482.1 (BPV1), M20219.1 (BPV2), JQ798171.1 (BPV13), and KR868228.1 (BPV14)²⁶ (Table 3).

The positive controls included BPV-1 DNA from a zebra sarcoid (gifted by Dr. Altamura, University of Naples, Italy), BPV-2 clone DNA (gifted by Dr. A. Venuti, IRCSS Regina Elena, National Cancer Institute, Rome, Italy), and BPV-13 and BPV-14 DNA from bovine bladder tumors from our laboratories^{6,14}. A BPV-negative sample and non-template control were included in each run.

Each sample was analyzed in triplicate to ensure accuracy. Samples were considered BPV-positive if at least three droplets containing BPV amplicons were present, consistent with the criterion for PV infections in human and veterinary medicine^{25,26,51}. Furthermore, samples with < 20 positive droplets were re-analyzed to ensure that these low copy number samples were not caused by cross-contamination. Sterile water served as a no-template control. Data acquisition and analyses were performed using the CFX Maestro software (Bio-Rad Laboratories).

Statistical analyses

The Friedman test was used to compare the median viral load of all bovine δ PV genotypes. Furthermore, to determine the significance relative to the number of copies of BPV DNA detected, the Friedman test was used after adjusting for Bonferroni multiple-comparison correction of the median. The Mann–Whitney U test was used to evaluate the viral load of single and multiple BPV infections. In all comparisons, statistical significance was defined by a P value < 0.05. Statistical analyses were performed using RStudio[®] software (version 4.2; The RStudio, Boston, MA, USA).

Data availability

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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Author contributions

A.C., G.F.: methodology; acquisition and analysis of the data; A.B., R.P., M.N.: visualization and statistical analysis; S.B. and S.R.: conceptualization, writing (original draft) S.R.: supervision, validation, funding acquisition, writing (review & editing). All authors reviewed the results; they approved the final version of the manuscript and declared that the content has not been published elsewhere.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics declaration

The animal collection and handling and tissue sampling procedures were performed in accordance with the ethics guidelines of the Veterinary University Vienna and Austrian Law. Animal studies performed in Naples were approved by the Institutional Animal Care and Use Committee (Protocol PG/2024/0023599, Naples University Federico II). Permission to collect samples was obtained from the animals' owners who were previously informed and in agreement with the purpose and methods used.

Additional information

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