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ORIGINAL ARTICLE

Screening for bovine papillomavirus type 13 (BPV13) in a European population of sarcoid-bearing equids

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Abstract

Background: Bovine papillomavirus types 1 and 2 (BPV1 and BPV2) are accepted aetiological agents of equine sarcoids. Recently, genetically similar BPV13 has been identified from equine sarcoids in Brazil.

Objectives: To determine whether BPV13 DNA can be also found in sarcoid-affected horses in Austria, and donkeys in Northern Italy and the UK, and should hence be considered in the context of vaccine-mediated sarcoid prevention.

Study design: Cross sectional study.

Methods: A total of 194 archival, equine and asinine DNA isolates derived from confirmedly delta-BPV-positive tumours were subjected to quality control by photometric analysis and equine beta-actin PCR. Isolates with DNA concentrations >0.9 ng/μl and confirmed PCR-compatibility (n = 135) were subsequently screened for the presence of BPV13 DNA using BPV13-specific PCR primers for amplification of a 771 bp region comprising the BPV13 E5 gene.

Results: BPV13 E5 PCR scored negative for all 135 samples. Included positive, negative and no-template controls yielded anticipated results, thus confirming reliability of obtained data.

Main limitations: Moderate number of tested tumour DNA extracts (n = 135; equivalent to 127 tumour-affected equids).

Conclusions: Despite its moderate size, the sample was considered representative enough to suggest a low occurrence of BPV13 in Austria, as it randomly comprised equine patients of different breed, age, gender, and European provenience. BPV13 was not associated with tested sarcoids in rescued donkeys originating from several other European countries. Large-scale BPV13 screenings are necessary to allow for a more precise estimation of the prevalence and distribution of BPV13 infections in European equids suffering from sarcoid disease.

KEYWORDS

bovine papillomavirus, BPV13, DNA, donkey, horse, PCR, sarcoid

The abstract is available in Portuguese in the Supporting Information section of the online version of this article.

1 | INTRODUCTION

It is widely accepted that sarcoid development and progression are driven by bovine papillomavirus type 1 and/or 2 (BPV1, BPV2) infection. BPV1 and BPV2 are members of the *Papillomaviridae*, a family of small icosahedral viruses that consist of a non-enveloped capsid harbouring a circular, double-stranded DNA genome.[1,2] The latter can be divided into an early (E), a late (L), and a noncoding long control region (LCR). In case of BPV1, BPV2, and BPV13, the early region comprises open reading frames (ORF) for the three oncogenes E5, E6, and E7, which assure maintenance of infection and induce transformation of normal to neoplastic cells.[3] Most papillomaviruses (PVs) are strictly species-specific and have a pronounced tropism for cutaneous and/or mucosal keratinocytes. The differentiation programme of these cells allows PVs to undergo a productive life cycle resulting in the generation and shedding of new infectious virions.[4] Delta-(δ-) papillomaviruses such as BPV1 and BPV2 are an exception to this rule in that they also infect dermal fibroblasts and—perhaps for this reason—have a wider host range including equids.[2] In bovine warts, BPV1 and BPV2 can be found in the epidermis and dermis. In the epidermis, infection is productive. In the dermis, however, BPV types 1 and 2 reside in fibroblasts as multiple extrachromosomal elements termed episomes, to assure genome maintenance.[2,4] In sarcoid-bearing horses, BPV1/2 infection predominantly involves dermal fibroblasts and is episomal in these cells.[5] Viral episomes replicate in synchrony with cell division.[4] This efficient form of PV amplification and the ability of BPV1 and BPV2 to establish latent episomal infection also in apparently healthy skin[6-8] likely explain the frequently reported onset, progression or reoccurrence of sarcoids following accidental or iatrogenic trauma.[9,10] The role of trauma in (re-) activation of PV-induced disease is widely accepted today.[11]

In 2013, Lunardi et al[12] identified a new δ-papillomavirus, ie BPV13, in papillomas of cattle in Brazil. Genetic characterisation of BPV13 revealed a high similarity to BPV2 (genetic homology >91%) and BPV1 (genetic homology >87%) and an identical genomic organisation. Hence, it was concluded that BPV13 possesses all the necessary features for the induction of fibropapillomas.[2,12-14] This conclusion was also supported by the detection of BPV13 DNA in sarcoids affecting two Brazilian horses.[15] More recently, the presence of BPV13 DNA was also reported for bovids and ovids in other regions of the world[16-19] demonstrating that the incidence of BPV13 is not limited to South America and supports the concept that BPV1, BPV2 and BPV13 have a similar pathogenic role.

Immunisation of horses with BPV1 L1 virus-like particles (VLPs) is safe and confers long-standing protection from experimental BPV1 and BPV2 infection and associated pseudo-sarcoid development.[20-23] It is still unclear whether BPV13 has a significant aetiopathogenetic role in equine sarcoid disease and hence deserves consideration in the context of prophylactic vaccine development.[21,24] We assessed archival sarcoid DNA extracts originating from equine patients in Austria for the presence of BPV13 DNA to obtain a first impression on the occurrence of BPV13 in this country. We also included DNA extracts derived from sarcoid-bearing donkeys originating from several European countries, to investigate whether BPV13—such as BPV1 and BPV2—may be found in this equid species.

2 | MATERIALS AND METHODS

The material studied included: (a) 155 archival DNA extracts prepared between 2007 and 2019 from tumour tissue, scrapings, swabs, or dandruff, or from perilesional hair roots of diagnosed sarcoids and sarcoid-like lesions (inflammatory skin conditions) affecting 155 horses in the catchment area of Vienna, Austria[25]; (b) 21 archival DNA extracts prepared from asinine tumour material obtained from 18 sarcoid-affected donkeys at several farms of the Donkey Sanctuary in Southern England, UK, between 2017 and 2019; and (c) 15 archival DNA extracts prepared from asinine tumour/peritumoural scrapings obtained from nine sarcoid-affected donkeys and a mule at the Italian subsidiary of the Donkey Sanctuary, ie the "Rifugio degli Asinelli" in Biella, Piedmont, in 2011 and 2012[26] (Table S1). In the context of this study, the catchment area of Vienna comprised Eastern Austria, Southern Czech Republic, Western Slovakia, and Western Hungary. Sarcoid-affected donkeys were initially rescued by the charity from different European countries, as specified in Table S2. All DNA extracts stored at −20 or −80°C, had previously tested positive by PCR using BPV1/2 consensus primers 5'E5 and 3'E5 for the amplification of a 499bp region comprising the E5 open reading frame (ORF) ("E5 PCR").[7,25,26] We designed this primer pair in 2005, long before the discovery of BPV13 (SB, personal communication). Recent BLAST alignment (https://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that primer sequences were 98 to 100% homologous to the corresponding genomic regions of BPV13. This implies that we may have unknowingly detected BPV13 DNA from sarcoids in the past, at least in cases where PCR products were not sequenced to identify the amplified BPV type, ie BPV1, 2, or 13.

To address this possibility, we first assessed PCR-compatible integrity of archival BPV1/2 E5 PCR-positive DNA isolates by (a) determining respective DNA concentrations using a NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer (ThermoScientific^{TM}, Vienna, Austria), and by (b) beta-actin PCR according to an established protocol.[27] DNA extracts with a minimum concentration of 0.9 ng/µl and scoring positive by beta-actin PCR as evidenced by gel electrophoresis and ethidium bromide staining were subsequently screened for the presence of BPV13 DNA using type-specific primers.

For primer design, a 1 kbp region of the BPV13 genome containing the E5 ORF was aligned to corresponding BPV1 and BPV2 DNA sequences using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify highly BPV13-specific DNA sequences upstream and downstream of the E5 ORF. Based on these sequences, the following primers were designed for amplification of a 771 bp region comprising the BPV13 E5 ORF: 5´BPV13-3682E5 (5´-ctgcaccaccacttggttta ctgtg-3´), and 3´BPV13-4452E5 (5´-gctagactgctagtggaccctgat-3´). Numbers in the primers' designations refer to the first (3682) and the

last nucleotide (4452) of the expected 771 bp amplification product, respectively, according to the GenBank sequence MF327274 (Figure 1).

Given that no wild-type BPV13 DNA was available as PCR-positive control (+c), plasmid vector pEX-A128 containing synthetic dsDNA (335 bp) corresponding to BPV13 nucleotide positions 3671-3830 and 4291-4465 according to MF327274 was generated (Eurofins Genomics, Vienna, Austria). Accordingly, resulting control plasmid pEX-A128-BPV13-E5-335 did not harbour the BPV13 E5 ORF, but adjacent upstream and downstream regions containing the BPV13 primer binding sites for amplification of a 311 bp product (Figure 1). DNA isolated from scrotal skin of sarcoid- and BPV1/2-free horses served as negative control (−c), and sterile water as no-template control (ntc) in all reactions. BPV13-screening was carried out in a step-wise approach, with every PCR being performed for ten sarcoid DNA samples, the positive, the negative and the no-template control, to minimise the risk of contaminations. BPV13 PCRs were carried out in 20 µl volumes containing 11.8 µl sterile water, 5 µl 5× buffer HF (ThermoScientific™), 0.6 µl DMSO (Sigma-Aldrich), 200 µM each of dNTPs (ThermoScientific™), 100 pmol each of 5´ BPV13-3682E5 and 3´ BPV13-4452E5 (Eurofins Scientific), 0.1 U Phusion Hotstart DNA polymerase (ThermoScientific™) and 1 µl of the template. The reactions were run in a Life Eco thermocycler (Biozym). The PCR programme consisted of an initial denaturation step at 98°C for 2 min, followed by 45 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. Amplification products (16 µl) were analysed by electrophoresis on 1.5% Tris-EDTA gels and visualised by ethidium bromide staining using a FluorChem FC3 imaging system (Biozym). Five µl of GeneRuler™ 100bp DNA Ladder (ThermoScientific™) per gel served as molecular weight marker.

Two amplicons of anticipated size were gel-extracted using a QIAEX II Gel Extraction Kit according to manufacturer instructions (Qiagen), re-amplified as described above, gel-extracted again and subjected to bidirectional sequencing (Eurofins). Resulting DNA sequences were identified by computed sequence alignment using BLAST.

Given that no BPV13 DNA was detected from the 135 DNA extracts derived from putatively BPV1/2 E5-positive sarcoids and sarcoid-like lesions, we further validated these data to avoid the possibility of presenting false-negative results. In a first step, we designed an additional positive control consisting of plasmid pEX-A128 harbouring a 900 bp region of BPV13 (MF327274 positions 3621-4520) that comprised the E5 ORF and the binding sites for BPV1/2 E5 primers and BPV13-specific primers described above (Eurofins Genomics; Figure 2). We then diluted the resulting plasmid pEX-A128-BPV13-E5-900 from 10^6 to 0 copies/µl equine fibroblast DNA and subjected this dilution series to BPV1/2 E5 PCR. Sarcoid DNA, equine fibroblast DNA and sterile water were included in the reaction as positive, negative and no-template controls. PCR was carried out as described above, with an amplification programme consisting of an initial denaturation step at 95°C for 5 min, followed by 45 amplification cycles (95°C for 15 s, 67°C for 30 s, 72°C for 30 s), and a final elongation step at 72°C for 5 min. In a subsequent step, we subjected the same dilution series to BPV13 PCR as described above. Finally, we repeated BPV13 PCR for 10 randomly selected sarcoid DNA aliquots. Plasmid pEX-A128-BPV13-E5-900 (10^6 copies) diluted in equine fibroblast DNA served as positive control in the reaction, equine fibroblast DNA and sterile water as negative control and ntc. All PCR products (16 µl amplicon each) were analysed by gel electrophoresis and subsequent ethidium bromide staining.

3 | RESULTS

In a first step, the 155 equine and 36 asinine BPV1/2-positive tumour DNA samples were assessed by spectrophotometry revealing DNA concentrations ranging between 15.162 µg and <0.9 ng per µl. Nine equine DNA isolates with concentrations <0.9 ng/µl were excluded. The remaining 146 equine and 36 asinine DNA extracts were subjected to standard beta-actin PCR that yielded amplicons of expected size (624 bp) from 99/146 (68%) equine, and 36/36 (100%) asinine DNA isolates (Table S1).

The beta-actin PCR-positive 135 DNA isolates were then subjected to BPV13 screening. In two cases, BPV13 PCR yielded weak bands of about 750-800 bp in size, whereas all other samples clearly scored negative. Positive (+c; pEX-A128-BPV13-E5-335) and negative (−c; ntc) results obtained for included controls testified for technical accuracy of obtained data. The two amplicons of anticipated size were re-amplified by BPV13 PCR and then sequenced bidirectionally. Subsequent sequence alignment identified these products as amplified bacterial DNA (Table S1).

To validate obtained BPV13 PCR-negative results, a second plasmid harbouring a 900 bp BPV13 genomic region comprising the E5 ORF was generated (Figure 2). Subsequent BPV1/2 E5 PCR from plasmid dilution series yielded expected 499 bp amplicons for dilutions 10^6-10, confirming that these primers not only recognise corresponding E5–spanning sequences of BPV1 and BPV2 but also BPV13. Anticipated results were obtained for the positive, negative and no-template controls (Figure 3). Subsequent BPV13 PCR from this dilution series was as sensitive as BPV1/2 E5 PCR, with dilutions 10^6-10 scoring positive (Figure 4A). Finally, BPV13 PCR repeated for ten randomly selected sarcoid DNA templates reproduced the negative results initially obtained for these samples, whereas the new plasmid control included in the reaction scored positive, as reflected by a prominent DNA band (Figure 4B).

4 | DISCUSSION

Equine sarcoids have worldwide occurrence, accounting for 12.9-67% of all equine tumours.[28] Similarly, donkeys and mules are commonly affected by sarcoid disease.[14] Although they do not metastasise, sarcoids can progress from single mild to multiple severe clinical types affecting a substantial proportion of the integument, especially upon trauma.[14] Despite considerable advances in

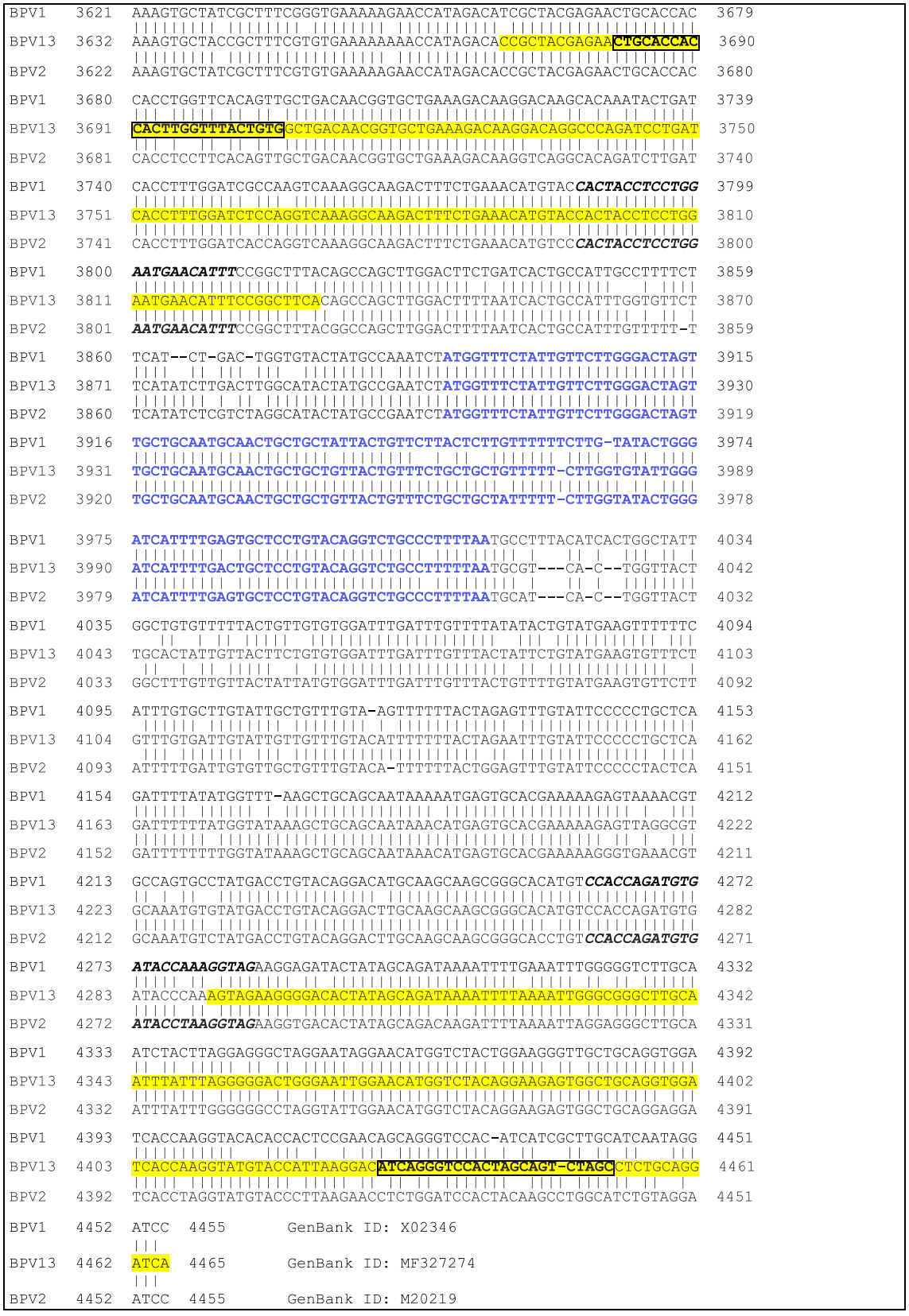

FIGURE 1 Alignment of the E5 ORF and adjacent regions of BPV1, BPV2 and BPV13 for design of BPV13-specific primers and the BPV13 DNA insert for plasmid pEX-A128-BPV13-E5-335. In bold with frame: BPV13 E5 primers: In bold and italics: BPV1/2 E5 primers[22]; In bold and blue: E5 ORF; highlighted in yellow: synthetic BPV13 DNA sequence used as positive control (Eurofins)

CTAACCAGGT AAAGTGCTAC CGCTTTCGTG TGAAAAAAAA CCATAGACAC CGCTACGAGA ACTGCACCAC
CACTTGGTTT ACTGTGGCTG ACAACGGTGC TGAAAGACAA GGACAGGCCC AGATCCTGAT CACCTTTGGA
TCTCCAGGTC AAAGGCAAGA CTTTCTGAAA CATGTAC***CAC TACCTCCTGG AATGAACATT TCC***GGCTTCA
CAGCCAGCTT GGACTTTTAA TCACTGCCAT TTGGTGTTCT TCATATCTTG ACTTGGCATA CTATGCCGAA
TCTATGGTTT CTATTGTTCT TGGGACTAGT TGCTGCAATG CAACTGCTGC TGTTACTGTT TCTGCTGCTG
TTTTTCTTGG TGTATTGGGA TCATTTTGAC TGCTCCTGTA CAGGTCTGCC TTTTTAATGC ATCACTGGTT
ACTTGCACTA TTGTTACTTC TGTGTGGATT TGATTTGTTT ACTATTCTGT ATGAAGTGTT TCTGTTTTTG
ATTGTATTGT TGTTTGTACA TTTTTTACTA GAATTTGTAT TCCCCCTGCT CAGATTTTTT ATGGTATAAA
GCTGCAGCAA TAAACATGAG TGCACGAAAA AGAGTTAGGC GTGCAAATGT GTATGACCTG TACAGGACTT
GCAAGCAAGC GGGCACATGT ***CCACCAGATG TGATACCCAA AGTAG***AAGGG GACACTATAG CAGATAAAAT
TTTAAAATTG GGCGGGCTTG CAATTTATTT AGGGGGACTG GGAATTGGAA CATGGTCTAC AGGAAGAGTG
GCTGCAGGTG GATCACCAAG GTATGTACCA TTAAGGACAT CAGGGTCCAC TAGCAGTCTA GCCTCTGCAG
GATCAAGAGC TGGAGCTGCT GCAGGTACTC GAAGCAGCAT CACCACAGGC ATCCCACTTG

FIGURE 2 BPV13 sequence inserted into pEX-A128 for generation of plasmid pEX-A128-BPV13-E5-900 as additional positive control. Highlighted in yellow: BPV13 E5 primer binding sites; highlighted in blue: BPV1/2 E5 primer binding sites; highlighted in red: primer mismatches; highlighted in green: start and stop codon of the BPV13 E5 gene

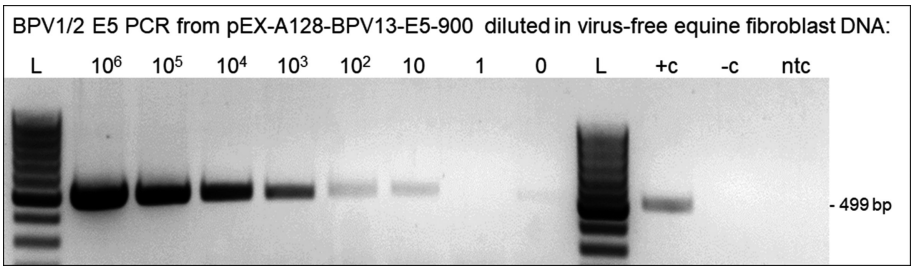

FIGURE 3 Consensus BPV1/2 E5 PCR is also suited for detection of BPV13. 10^6 - 0: pEX-A128-BPV13-E5-900 copy numbers. L: GeneRuler™ 100bp DNA Ladder (ThermoScientific™); +c: sarcoid DNA; −c: virus-free equine fibroblast DNA; ntc: no-template control (sterile water); −499 bp: expected amplicon size

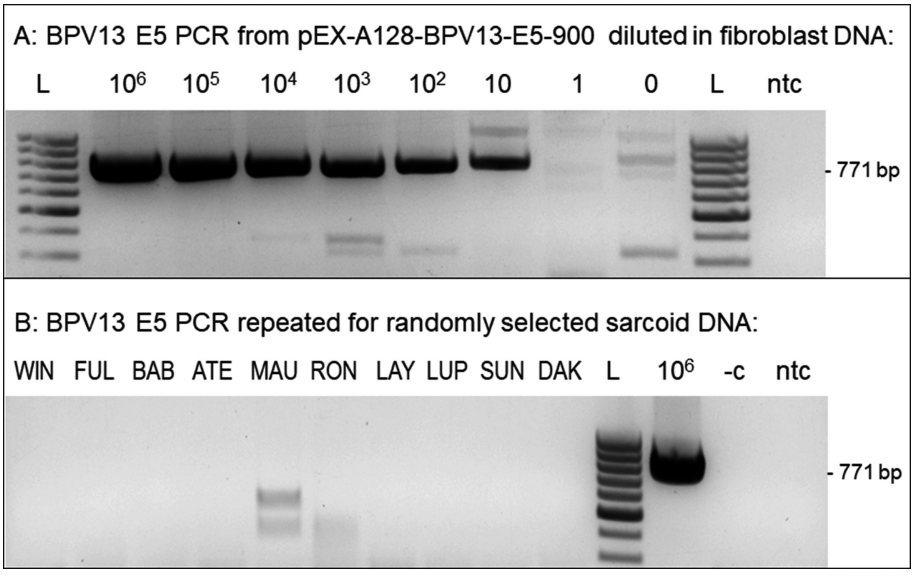

FIGURE 4 BPV13 PCR is highly sensitive and yields reproducible results. (A) BPV13 PCR detects as little as 10 copies of pEX-A128-BPV13-E5-900. 10^6 - 0: pEX-A128-BPV13-E5-900 copy numbers. L: GeneRuler™ 100bp DNA Ladder (ThermoScientific™); ntc: no-template control (sterile water); −771 bp: expected amplicon size. (B) BPV13 PCR repeated for 10 randomly selected samples with pEX-A128-BPV13-E5-900 diluted in horse DNA as positive control reproduces initially obtained data. WIN-DAK: tumour DNA isolates according to Table S2. L: GeneRuler™ 100bp DNA Ladder (ThermoScientific™); 10^6: equine fibroblast DNA containing 10^6 copies of pEX-A128-BPV13-E5-900 as positive control; −c: equine fibroblast DNA; ntc: no-template control (sterile water); −771 bp: expected amplicon size

sarcoid management, therapy is still challenging and ineffective in a considerable number of cases.[29] Hence, substantial efforts are made to establish more effective sarcoid therapeutics, notably immunotherapeutics[30] and BPV1 L1 VLP-based vaccines for sarcoid prevention.[21] In this context, new potential aetiological agents of sarcoid disease need to be considered.

BPV1 and BPV2 DNAs are detected from sarcoids all over the world, with BPV1 prevailing in central and northern Europe as well as the eastern USA, whilst BPV2 predominates in Southern Europe and the Western United States.[8] In addition, genetic BPV1 variants have been identified and initially interpreted as "equine variants".[31,32] However, recent data indicate that these variants have rather a geographical origin than being species-specific.[33] Based on these observations, the first identification of BPV13 from papillomas affecting Brazilian cattle, and the detection of BPV13 DNA from sarcoids in two Brazilian horses[12,15] led to the speculation that BPV13 may represent a South American subtype of BPV1 or BPV2. However, subsequent detection of BPV13 DNA from bovids in Southern Italy and Iraq[17,19] refutes this theory and rather suggests a worldwide incidence of this PV type, as is the case for BPV1 and BPV2.

This assumption further motivated us to screen DNA from sarcoids and putative sarcoids for the presence of BPV13 DNA. To this aim, we designed a primer pair that proved highly BPV13-specific, as it failed to anneal to corresponding BPV1 or BPV2 DNA regions confirmedly present in some of the samples,[7,25] but yielded prominent bands of expected size (311 bp) for the positive control (synthetic 335 bp dsDNA harbouring the primer binding sites; Figure 1). Prior to BPV13 screening, PCR-compatible integrity of DNA isolates was assessed by photometric measurement and beta-actin PCR. Failure to detect this housekeeping gene from some equine sarcoid DNA isolates was not due to long storage at −20°C (up to 10 years), but to repeated thawing, use, and freezing, as revealed by laboratory records.

All tumour DNA isolates tested in this study had scored positive by consensus BPV1/2 E5 PCR in previous experiments.[7,25] Recent primer alignments indicated that these primers can also be used for amplification of the corresponding BPV13 sequence, so that we may have unknowingly detected BPV13 from some of these samples in the past. To test this assumption, plasmid pEX-A128-BPV13-E5-900 (Figure 2) was serially ten-fold diluted in virus-free equine fibroblast DNA and subjected to consensus BPV1/2 E5 PCR. As expected on the basis of primer alignments results, the reaction scored positive for most dilutions, with 10 copies pEX-A128-BPV13-E5-900 representing the detection limit against the background of equine DNA used as diluent (Figure 3).

In fact, all 135 tumour DNA isolates tested negative by BPV13 PCR. To address the authenticity of data, we further assessed the sensitivity of the assay by BPV13 PCR from pEX-A128-BPV13-E5-900 dilution series. Positive results obtained for 10^6 to 10 copies of plasmid against the background of horse DNA confirmed the sensitivity of the reaction (Figure 3A).

Due to budgetary restrictions, pEX-A128-BPV13-E5-335, ie a plasmid allowing for amplification of a BPV13-specific 311 bp product (in contrast to the 771 bp amplicon expected for positive samples), was used as positive control throughout BPV13 amplification reactions. To rule out the rather improbable possibility that BPV13 PCR might have yielded positive results for the control, but false-negative results for some samples due to different amplicon sizes, BPV13 PCR was repeated for 10 randomly selected BPV13-negative sarcoid samples, with pEX-A128-BPV13-E5-900 diluted in horse DNA serving as positive control. The reaction reproduced initially obtained data, as expected by reason of its high sensitivity and specificity (Figure 4B).

Failure to detect BPV13 DNA from the 135 tumour DNA isolates does not rule out the possibility that BPV13 is occasionally present in equid sarcoid patients from Austria, the UK and other European countries represented in this study (Table S2). However, despite its moderate size, the sample is considered representative, as it randomly comprised tumour DNA extracts from horses and donkeys of different age, gender, breed and origin prepared between 2007 and 2019. Based on our findings, the higher homology of BPV13 to BPV2 (>91%), than to BPV1 (>87%), and the so far reported detection of BPV13 in Brazil, Southern Italy and Iraq, it appears possible that BPV13 may prevail in southern geographic regions and perhaps has evolved from BPV2. To our knowledge, this is the first study addressing the presence of BPV13 in a substantial number of sarcoids and sarcoid-like lesions, ie cases of inflammatory skin conditions. However, large-scale screenings and in-depth molecular biological analyses are necessary to reach definitive conclusions as to the worldwide prevalence and distribution of BPV13 in sarcoid-affected (and healthy) equids, and the impact of BPV13 infection on sarcoid development and progression.

ETHICAL ANIMAL RESEARCH

Research ethics committee oversight is not currently required by this journal: the study was performed on material collected during clinical procedures and material collected non-invasively.

INFORMED CONSENT

For material collected specifically for this study, animal owners or their representatives gave consent for its inclusion. For archived material submitted for diagnostic purposes, explicit owner informed consent for inclusion of animals in this study was not stated.

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CONFLICT OF INTERESTS

No competing interests have been declared.

AUTHOR CONTRIBUTIONS

C. Jindra contributed to study design, study execution, and data analysis and interpretation. A.-K. Kamjunke contributed to study execution and data analysis. S. Jones contributed to the study execution and preparation of the manuscript. S. Brandt contributed to study design, data analysis and interpretation, and preparation of the manuscript. All authors had full access to all data and take responsibility for the integrity of the data and the accuracy

of the data analysis. All authors approved the final version of the manuscript.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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