SEQUENCE VARIANTS OF E5 ORFs BOVINE PAPILLOMAVIRUS DETECTED IN FORMALIN–FIXED AND PARAFFIN–EMBEDDED TISSUES OBTAINED FROM EQUINE SARCOIDS

ANNA SZCZERBA-TUREK, JAN SIEMIONEK, WOJCIECH SZWEDA, AGATA BANCERZ-KISIEL, ANDRZEJ RAS, AND TADEUSZ ROTKIEWICZ

Department of Epizootiology, 1Department of Animal Reproduction with Clinic, 2Division of Pathological Anatomy, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, 10-718 Olsztyn, Poland

a.szczerba@uwm.edu.pl

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Abstract

The aim of the study was to identify the bovine papillomavirus (BPV) types associated with equine sarcoi ds observed in Polish horses, during 2001–2004. The samples of skin tumours were formalin-fixed and paraffin-embedded. Out of 14 tumours obtained from 10 horses, 11 were diagnosed as fibroblastic sarcoid, two as verrucose sarcoid, and one as epidermal cystoid. Using PCR, the presence of E5 open reading frames (ORFs) fragment BPV-1/BPV-2 were shown in all specimens obtained from archival tissues. Sequence variants of E5 ORFs confirm that European variants of BPV-1 occur in fibroblastic and verrucose equine sarcoi ds in Poland.

Key words: horse, bovine papillomavirus, sarcoi ds, PCR, diagnosis, phylogenetic analysis, Poland.

Equine sarcoi ds are the most common skin tumours in the horse (12, 15). They affect horses of all ages, types and coat colour, without obvious sex predilection, and they are commonly encountered worldwide. Sarcoi ds are recognised as having six different clinical types: verrucose, fibroblastic, occult, nodular, mixed, and malignant (9, 10). All the types may occur at any skin site. The etiology of the equine sarcoi d is not clearly understood but the association between BPV-1 and BPV-2 and equine sarcoi d is well documented (2, 6). With a number of molecular techniques, including Southern blot hybridisation and polymerase chain reaction (PCR), BPV-DNA can be detected in 86%-100% of equine sarcoi ds (7, 13, 16). BPV-1 and BPV-2 belongs to Papillomaviridae family, the same as human papillomavirus type 16 and 18 (HPV-16 and 18), the main etiological agent of cervical cancer in women worldwide. On the National Centre for Biotechnology Information, 10 whole BPV genomes and up to 100 whole HPV genomes are sequenced. All papillomaviruses (PV) are strictly species-specific and, even under experimental conditions, do not infect other species. Equine sarcoi ds are the result of a natural cross-species infection by BPV-1 and BPV-2. The paper aimed at the identification of bovine papillomavirus types associated with equine sarcoi ds diagnosed in Polish horses.

Phylogenetic tree (unrooted) containing the whole sequence of 20 PV types is shown on Fig. 1. The GenBank accession numbers of their sequences are: NC001522 (BPV-1); M20219 (BPV-2); NC004197 (BPV-3); X05617 (BPV-4); NC004195 (BPV-5); AJ620208 (BPV-6); NC007612 (BPV-7); NC009752 (BPV-8); NC001356 (HPV-1); NC001352 (HPV-2); NC001593 (HPV-53); NC002644 (HPV-71); NC012123 (EqPV-2); NC003748 (EcPV-1); NC001541 (CRPV); NC001619 (COPV); NC001789 (OvPV-1).

Material and Methods

Sample collection. We selected for the study 14 archived formalin-fixed and paraffin-embedded (FFPE) tissue blocks of equine sarcoi d with accompanying slides stained with haematoxylin and eosin, prepared between 2001 and 2004 in the Division of Pathological Anatomy. The specimens were obtained from 10 horses.

DNA extraction. We cut sections from each tissue block. Deparaffinisation was carried out by adding 1 ml of xylene to the microtube containing the sections (two changes for 60 min each), followed by 100% ethanol for 20 h, 70% ethanol for 60 min, 50% ethanol for 20 h, and 30% ethanol for 4 h.
After the washing step with water for 30 min, DNA was extracted from tissue fragments. The DNA was extracted with the use of the Genomic Mini for DNA extraction (A&A Biotechnology, Poland) in accordance with the manufacturer’s instructions, except that we incubated tissue sections with lysis buffer overnight until all tissue fragments were completely dissolved. The DNA concentration was determined spectrophotometrically using BioPhotometr (Eppendorf, Germany). The samples were stored at -20°C.

**PCR procedure.** The purpose of PCR was to amplify fragment E5 ORF BPV-1 and/or fragment E5, E25 ORFs BPV-2. The final volume of PCR mixture was 25 µl and the final concentration of magnesium
chloride was 4 mM. Reactions were performed in the presence of Taq DNA polymerase (Fermentas, Lithuania), according to the manufacturer’s instructions. Amplification was carried out in a mastercycler (Eppendorf, Germany). Cycling conditions were as follows: 1x 94°C for 10 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final extension of 72°C for 10 min. Plasmids containing the entire BPV-1 or BPV-2 genomes, obtained from Professor M. Saveria Campo from the Institute of Comparative Medicine, University of Glasgow, U.K., were used as a positive control. Oligonucleotide primers for BPV-DNA used in this reaction, of types 1 and 2 BPV, were published by Teifke et al. (18). The primer sequences were E5L2up 5’ CAA AGG CAA GAC TTT CTG AAA CAT 3’ and E5L2lo 5’ AGA CCT GTA CAG GAG CAC TCA A 3’, both synthesised in the Institute of Biochemistry and Biophysics, Polish Academy of Sciences (IBB-PAN). The primer set amplified a 242 base pair (bp) fragment of the E5 ORF BPV-1, at nucleotide positions 3,760 – 4,003 (GenBank accession number X02346), and 245 bp fragment of the E5, E25 ORFs BPV-2, at positions 3,760 – 4,006 (GenBank accession number M20219). All manipulations were performed very carefully to avoid contamination and the negative control samples were analysed in between the sarcoid samples.

Separation and analysis of PCR products. The PCR products were separated in a 2% agarose gel in 1x concentrated TAE at a voltage of 5 V per 1 cm of gel. Into each well, 10 µl of reaction mixture and 2 µl of loading buffer 6X Loading Dye Solution (Fermentas, Lithuania) were loaded. The molecular weight of the obtained products was determined on the basis of molecular weight marker, which was GeneRuler™ 100 bp DNA Ladder (Fermentas, Lithuania). The gels were stained with ethidium bromide and the amplification products were visualised under UV light. The results of electrophoresis were archived using the gel documentation system GelDoc™ EQ (Bio-Rad, USA).

Phylogenetic analysis. PCR products of DNA BPV were cleaned using Clean-up (A&A Biotechnology, Poland) in accordance with the manufacturer’s instructions. Cleaned products of PCR were sequenced in the IBB PAN. The analysis of the results was done using BLASTN version 2.2.18. (1), BioEdit Sequence Alignment Editor, Clustal W methods (11). Phylogenetic analysis was done by DNADist – Neighbor phylogenetic tree methods, version 3.5c, using TreeView version 1.6.6. (14). Sequence data from the specimens were compared with the nucleotide sequence of other PVs in GenBank, by means of the blast algorithm.

Results

The histopathological and molecular examinations were performed on 14 skin lesions taken from 10 horses. Sarcoïds were diagnosed in 93% of skin lesions. The fibroblastic sarcoid was diagnosed in 85%. As the result of amplification of E5 ORFs BPV using PCR procedure, we detected the presence of selected gene fragment in all the specimens (Fig. 2).

Phylogenetic analysis demonstrated that 14 E5 ORFs of BPV-1 contained the same nucleotide substitution, A3939G, which had been previously detected in BPV-1 sequence isolated from donkey (16) and equine (7) sarcoïds, suggesting that this sequence variant is an European variant of BPV-1. The nucleotide sequences of 13 BPV E5 ORFs isolated from FFPE tissues were identical to the prototype sequence BPV-1 subtype III isolated in Switzerland and 1 BPV E5 ORFs was identical to the prototype sequence BPV-1 subtype I isolated in United Kingdom (Fig. 3). Our results confirm that European variants of BPV-1 occur in fibroblastic and verrucose equine sarcoïds in Poland.

![Fig. 2. Photomicrograph of an ethidium-bromide stained agarose gel of PCR products obtained with the use of a bovine papillomavirus primer set. Lane M - DNA size marker GeneRuler™ 100 bp DNA Ladder (Fermentas, Lithuania), lane 1 – negative control without viral DNA, lane 2 – positive control of BPV-1, lanes 3, 4, 5, 6, and 7 - BPV positive specimens.](image-url)
Discussion

BPV plays an important role in the etiology and pathogenesis of equine sarcoids. This is the first report of European variant of BPV-1 detected in equine sarcoids in Poland. In the current study we detected fragment of E5 ORF BPV-1 in all specimens. E5 ORF encodes E5 protein, which is the major oncoprotein of BPV, expressed in the deep layers of the epithelium. After histopathological examination, we diagnosed sarcoids in 93% of skin lesions, but using PCR procedure we revealed BPV-DNA in all the specimens. The exact manner of BPV infections of horses and the development of equine sarcoids has not been fully understood yet. Information about the presence of BPV-DNA in lesions different than sarcoids is not clear. Carr et al. (4, 5) and Brandt et al. (3) found BPV-DNA only in skin lesions recognised as sarcoids, but Chambers et al. (7) and Yuan et al. (19) detected BPV-DNA in sarcoids and in granulomatous dermatitis, ulcerative dermatitis, and eosinophilic perivascular dermatitis.

Different results of the presence of BPV-DNA in different skin lesions obtained from horses suggest that the investigations should have been continued.

In the current study we decided to work with FFPE tissues. FFPE tissues are one of the popular sources of diagnostic materials. Although the technique of DNA extraction from FFPE tissues is well documented, preparations from fixed tissues always exhibit certain limitations for PCR (8). They are often used as the source of nucleic acids for retrospective molecular analyses based on DNA amplification by PCR. However, it is known that nucleic acids from FFPE are much worse templates than those recovered from fresh tissues, but it is the most invaluable source of diagnostic material for studying pathogenesis of cancer and a variety of other diseases. The effect of formalin fixation on the efficiency of amplification is well known (17). In our study we did not know how long the tissues were fixed in formalin and the first trial gave no effect. Therefore, the time of hydration of archival tissues was elongated. The another problem with an
ampliﬁcation is the size of amplification products, if the amplicon is about 250 bp or less we do not have problems with the efﬁciency of PCR (8). The retrospective molecular analyses play an important role in molecular epidemiology, and the results of retrospective research allow us to certify proposals for the prospective study.

In conclusion, our results showed that in archival samples from equine sarcoids fragment of E5 ORFs BPV-1 occurs and this conﬁrms data from the literature, that in etiology and pathogenesis of equine sarcoid, BPV-1 is of great importance (3, 19, 20). The sequence of the BPV-1 E5 ORFs was identical in all cases to the sequence in the database (NCBI). In all samples we detected, European variants of BPV-1 and the nucleotide substitution in these variants were described previously by other authors (7, 16, 19, 21). There are many unaccountable facets of the relationship between equine sarcoid and BPV which remain to be elucidated, and our retrospective work conﬁrmed the main role of BPV-1 in the pathogenesis of equine sarcoids in Poland.

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References