

Detection of bovine papillomavirus in an equine cell line as a contaminant

Detectie van runderpapillomavirus in een paardencellijn als contaminant

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ABSTRACT

Bovine papillomaviruses (BPV) are members of the family *Papillomaviridae*. Papillomaviruses are usually species-specific and epitheliotropic. Delta-BPVs are an exception to this rule in that they can also infect fibroblasts and non-bovid ungulates. Cell cultures are essential for performing in vitro studies, analysis of virus biology, vaccine production, tissue engineering and toxicity testing. In this context, cell line contamination constitutes a significant problem. In this study, various cell lines (n=27) were assessed for potential BPV contamination. To this aim, DNA was extracted from cell cultures and then screened for the presence of papillomavirus L1 capsid gene DNA using a consensus polymerase chain reaction (PCR) system. Immunofluorescence (IF) staining was used for viral protein detection. Intriguingly, one cell line derived from equine dermis tested positive by PCR and subsequent IF staining for L1. Amplicon sequencing followed by computed L1 DNA sequence alignment led to the identification of a new putative BPV type, revealing the highest identities with Delta-BPV types 1 (76%) and 2 (73%). To the authors' knowledge, this is the first report on the presence of a putative BPV as a viral contaminant in cell cultures that possibly represents an unknown Delta-BPV.

SAMENVATTING

Boviene papillomavirussen (BPV) behoren tot de familie van *Papillomaviridae*. Papillomavirussen zijn meestal soortspecifiek en epitheliotroop. Delta-BPVs vormen een uitzondering op deze regel omdat ze ook fibroblasten en niet-boviene hoefdieren kunnen infecteren. Celcultuur is essentieel voor het uitvoeren van in-vitrostudies, analyse van virusbiologie, vaccinproductie, weefselmanipulatie en toxiciteitstesten. In deze context vormt cellulaire contaminatie een aanzienlijk probleem. In de voorliggende studie werden verscheidene cellijnen (n = 27) beoordeeld op mogelijke BPV-besmetting. Voor dit doel werd DNA geëxtraheerd uit een celcultuur en vervolgens gescreend op de aanwezigheid van papillomavirus L1-capsidegen DNA met behulp van een consensus-polymerasekettingreactie (PCR)-systeem. Immunofluorescentiekleuring (IF) werd gebruikt voor de detectie van virale eiwitten. Opmerkelijk was dat één cellijn afgeleid van paardendermis positief testte met PCR en met de daaropvolgende IF-kleuring voor L1. Amplicon-sequencing gevolgd door L1-DNA-sequentievergelijking leidde tot de identificatie van een vermoedelijk nieuw BPV-type dat de grootste gelijknissen met Delta-BPV-typen 1 (76%) en 2 (73%) vertoonde. Volgens de auteurs is dit het eerste rapport over de aanwezigheid van een vermeende BPV als een virale contaminant in celculturen die mogelijk een tot nu toe onbekende Delta-BPV vertegenwoordigt.

INTRODUCTION

Papillomaviruses (PV) are non-enveloped, icosahedral viruses containing a circular double-stranded DNA genome. They have the potential to establish a latent infection and cause tumor formation in cutaneous and/or mucosal epithelia. PVs are usually epitheliotropic host-specific viruses that are found in a wide range of vertebrate species (Nasir and Campo, 2008; Rector and Ranst, 2013). Alike all PVs, bovine papillomaviruses (BPVs) are members of the family *Papillomaviridae* (De Villiers et al., 2004; Lancaster, 1981). To date, twenty-four BPV genotypes are known, and are classified into the genera, *Deltapapillomavirus* (BPV 1, 2, 13, 14), *Epsilonpapillomavirus* (BPV 5, 8), *Dyoxipapillomavirus* (BPV 7), *Xipapillomavirus* (BPV 3, 4, 6, 9, 10, 11, 15), and *Dyokappapapillomavirus* (BPV 16, 22) (Ataseven et al., 2016; Bauermann et al., 2017; Da Silva et al., 2016). Interestingly, Delta-BPVs can also infect dermal fibroblasts and are less species-specific than other PVs. In addition to cattle, they also infect other ruminant species as well as equids. In bovids, BPVs mainly induce benign papillomas that regress spontaneously. In some cases however, several types such as BPV4 are involved in the development and progression of squamous cell carcinomas (Nasir and Campo, 2008). In equids, BPV-1 and BPV-2 are the causative agents of persistent, locally invasive skin lesions termed sarcoids, which are the most common tumor type in horses (Chambers et al., 2003).

Microbial contamination of cell cultures with bacteria, yeasts, viruses or mycoplasma, is the most common problem encountered in cell culture laboratories. Consequences include viral interference, misidentification, cell integrity damage, falsification of research results, mutations and health hazard to the laboratory personnel (Mirjalili et al., 2005). To increase the sensitivity of studies and the reliability of test results, and to reduce the waste of expensive biological reagents, such as enzymes, antibodies, or media, cell cultures need to be assessed for microbial contamination whenever applicable.

The authors have previously assessed cell culture systems and sera in their laboratory for contaminations with non-cytopathogenic pestiviruses and did not detect these viruses. In the present study, the screening of various, established primary cell lines that are routinely used in the laboratory for contamination with papillomaviruses, is reported. To the best of the authors' knowledge, this study is the first to describe the contamination of a cell line by a bovine papillomavirus (BPV).

MATERIALS AND METHODS

Cell lines originating from cattle, rabbits, horses, hamsters, cats, dogs, monkeys and humans were ob-

tained from the cell culture collection of the Department of Virology (Faculty of Veterinary Medicine of the Ankara University). Twenty-seven different cell lines, including five homologous cell lines from various sources, and fetal sera for media preparation were tested for papillomavirus contamination (Table 1). To this aim, cells were maintained at 37°C in a 5%-CO₂ atmosphere in Dulbecco's modified Eagle's medium (Biochrom AG, Berlin, Germany), containing 100 µg streptomycin and 100 IU penicillin per ml as well as 10% fetal bovine serum (Biochrom AG). After five days, cells were harvested and subjected to total DNA extraction according to the phenol:chloroform:isoamyl alcohol extraction method described by Sambrook and Russell (2001). The resulting DNA pellets were resuspended with 20 µl of deionized water. Respective DNA concentrations were determined using a Picodrop Microliter UV/Vis spectrophotometer (Picodrop, Cambridge, UK). Then, DNA isolates were stored at -20°C until use. Polymerase chain reaction (PCR) of papillomavirus was performed according to Forslund et al. (1999) using degenerate primers FAP59/64 (5'-TAACWGTIGGICAYCCWTATT-3' and 5'-CCWATATCWVHCATITCICCATC-3') for amplification of a 478-bp region within the PV *L1* capsid gene. Reactions were carried out in a total volume of 30 µl containing 5 U *Taq* DNA polymerase (MBI Fermentas, Waltham, MA, USA), 3 µl of 10XTaq Buffer (MBI), 25 mM MgCl₂, 10 pmol of each primer, 10 mM dNTP mix (MBI), 18.1 µl of sterile water (18 MOcm, AppliChem, Darmstadt, Germany), and 3 µl of extracted DNA. An initial denaturation step at 94°C for 10 minutes was followed by 40 cycles of DNA denaturation at 94°C for 50 seconds, primer annealing at 51°C for 55 seconds, and DNA elongation at 72°C for 50 seconds. Finally, reaction tubes were kept at 72°C for a further 10 minutes for final extension. PCR products were visualized in a UV-transilluminator following electrophoresis in 1%-agarose gels containing ethidium bromide. PCR products of expected size (478 bp) were gel-extracted using a Gene JET PCR purification Kit (Thermo Scientific, Waltham, MA, USA), and PCR fragments were sent to a commercial company (RefGen, Ankara, Turkey) for Sanger sequencing. The sequences obtained were aligned by using CLC Main Workbench v5.5 (CLC Bio, Aarhus, Denmark) and analyzed with MEGA 6.06 (Tamura et al., 2013). Maximum-likelihood trees were generated based on the Kimura two-parameter model with 1,000 bootstrap replicates (Figure 1). Partial sequences of the *L1* gene were submitted to the NCBI GenBank database and were assigned the accession number KY592382.

For the immunofluorescent assay, PCR-positive equine dermal (ED) cells, as well as NBL-6 cell line used as negative control, were seeded onto 24-well plates and grown for 48 hours (Figure 2). Then, cells were fixed with 4% formalin for 30 minutes at 4°C, permeabilized with 0.1% Triton X-100 in phosphate-

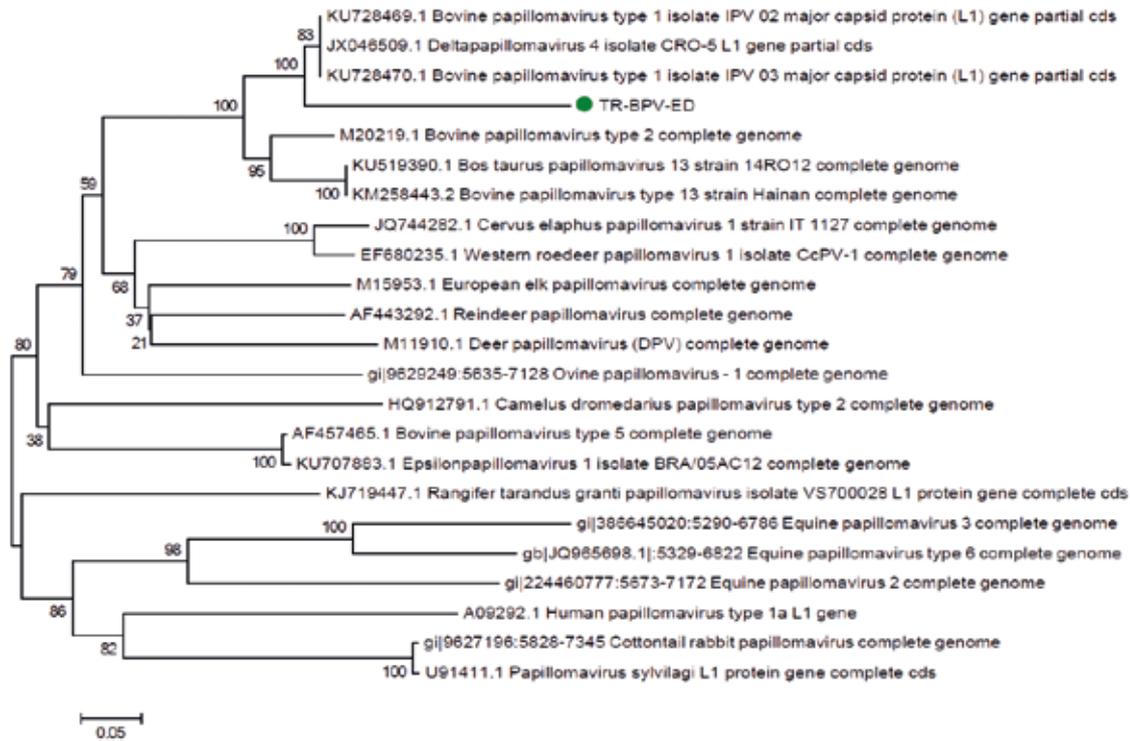


Figure 1. The comparison of known papillomavirus types with TR-BPV-ED based on partial L1 protein coding sequences using neighbor-joining method. Virus types represented are indicated by name following GenBank accession number. The optimal tree with the sum of branch length = 3.81460836 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated.

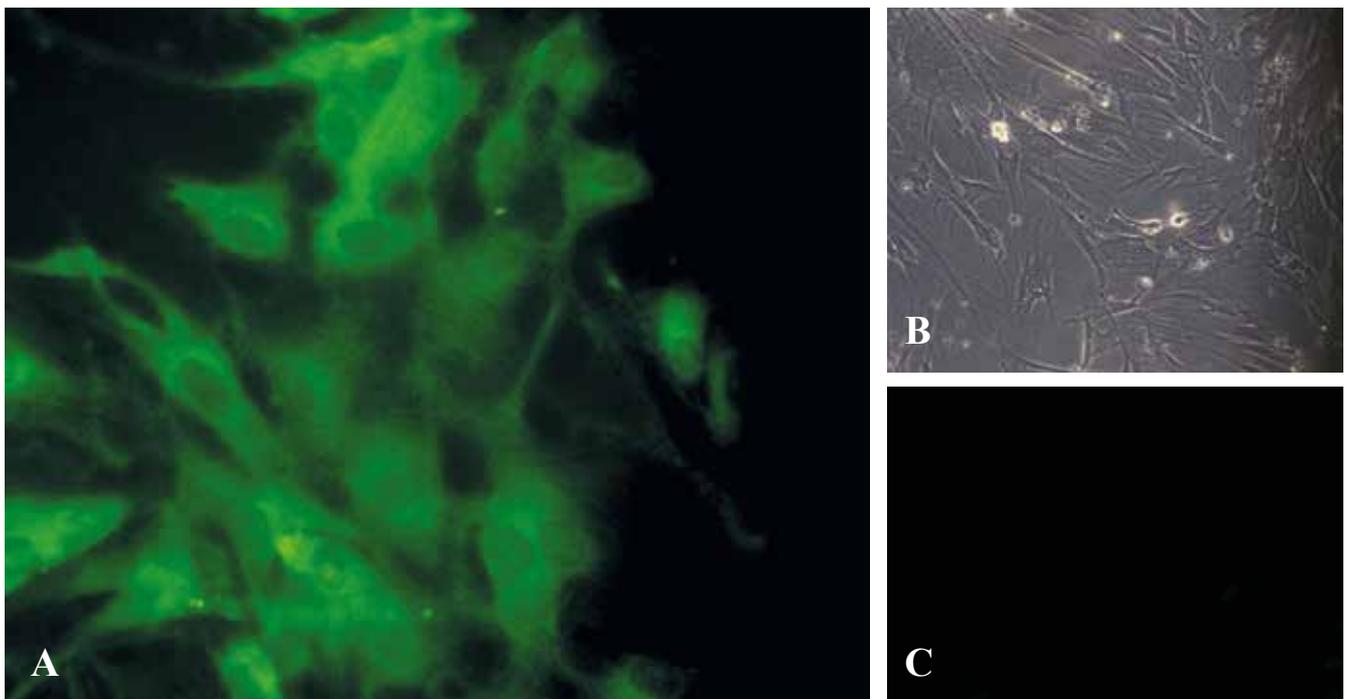


Figure 2. A. Immunofluorescence staining of ED cells infected with papillomavirus using BPV-1 E2 specific monoclonal antibody (20X). B. Image of ED cells by light microscope (20X). C. Image of NBL-6 cell line as a negative control.

Table 1. Investigated cells.

Cell line	Reference	Description
RK-13	ATCC® CCL-37™	European rabbit kidney cell line
MA104	ATCC® CRL-2378.1™	African green monkey kidney cell line
MDBK	ATCC® CCL-22™	Madin-Darby bovine kidney cell line
Vero E6	ATCC® CRL-1586™	African green monkey kidney cell line
NBL 6	ATCC® CCL-57™	Equine dermal fibroblast cell line
ED	Permanent cell line ¹	Equine dermal fibroblasts
MDCK	ATCC® CRL-2935™	Canine kidney cell line
CRFK	ATCC® CCL-94™	Crandell Reese feline kidney cell line
BHK-21	ATCC® CCL-10™	Baby hamster kidney cell line
HCT-8	ATCC® CCL-244™	Human colonic adenocarcinoma cell line
NEL-M1	Primary cell line ²	Human melanoma cells
SFT-R	CVCL-RIE 0043	Ovid thymus cell line
FCWF	ATCC® CRL-2787™	Feline whole fetus cell line
ECF-R	CLS ID 601469	Chicken embryo fibroblastoid cell line
FL	ATCC® CCL-62™	HeLa contaminant
HEL	ATCC® TIB-180™	Human erythroleukemia cell line
FEL	Primary cell line ³	Feline embryonic lung cells
SK-6	CCLV-RIE 0262	Swine kidney-6 cell line
FBLC	Primary cell line ⁴	Immortalized fetal bovine lung cells
PK-15	ATCC® CCL-33™	Swine kidney cells
FBKC	Primary cell line ⁵	Immortalized fetal bovine kidney cells
BT	Primary cell line ⁶	Bovine turbinate cell line

¹ Anonymous cell line from cell culture collection in the lab of the authors.

² DiSorbo D.M., et al. (1985).

³⁻⁶ Oğuzoğlu T.C. (2003-2006) (unpublished).

buffered saline (PBS) for 10 minutes at 4°C and blocked with 3% bovine serum albumin (BSA) in PBS for 30 minutes at room temperature. Subsequently, cells were incubated with anti-BPV-1 E2 monoclonal antibody 1H10 (Thermo Fisher Scientific, catalog # MA1-71539,) at a dilution of 1:1,000 in PBS containing 3% BSA at 4°C overnight. After washing, cells were incubated with Alexa Fluor Plus 488 goat anti-mouse IgG as a secondary antibody (Invitrogen, Thermo Fisher Scientific, catalog # A32723) at a dilution of 1:1,000 for 3 hours at 4°C. Immunofluorescence was visualized using the Zeiss Axio Observer microscope (Carl Zeiss, Jena, Germany).

RESULTS

Consensus PCR was used to screen 27 cell lines and sera for papillomavirus DNA. Overall, 26 cell lines and 6 batches of serum samples tested negative by this assay. In contrast, DNA extracted from ED cell line (initial concentration of 393.26 ng/ml) derived from equine dermis had scored positive. Consequently, the resulting amplicon was sequenced. Subsequent alignment with sequences deposited in the GenBank revealed this amplicon sequence to be 40.1–76.3% identical to various BPV strains.

Comparison of the sequence in the present study with other genomic papillomavirus sequences derived

from many different species, i.e. cow, camel, deer, elk, reindeer, caribou, sheep, horse, rabbit and human, was carried out (Figure 1). Divergence and percent identity are shown in Figure 3. Homology analyses revealed a 76.3% identity of the sequence of this study, termed TR-BPV-ED, to BPV-1 isolate CRO-5. This relationship represented the highest similarity among the compared sequences. This sequence also showed 71.8% identity with BPV-2, followed by a sequence identity of 70.2% with *Bos taurus* papillomavirus 13 strain 14RO12 and BPV-13 strain Hainan. BPV type 5 and *Epsilonpapillomavirus* 1 showed only 56.3% and 56.1% identity with TR-BPV-ED, respectively. Equine papillomavirus-2, equine papillomavirus-3, and equine papillomavirus-6 complete genomes shared identities of 44.8%, 40.1%, and 42.7%, respectively, with TR-BPV-ED. Genetic similarity with equine papillomavirus-3 amounted to 40.1%. Human papillomavirus type 1 shared 48.8% sequence identity with TR-BPV-ED.

Moreover, *Papillomavirus sylvilagi* and cottontail rabbit papillomavirus exhibited 49.8% and 50.0% identity, respectively. Ovine papillomavirus shared 55.2% identity with TR-BPV-ED. In regard to deer and camel papillomavirus strains, *Cervus elaphus* papillomavirus 1 strain IT 1127 and *Camelus dromedarius* papillomavirus type 2 shared sequence identities of 56.8% and 53.8% with TR-BPV-ED, respectively. European elk papillomavirus, reindeer pap-

Table 2. List of sequences used in the phylogenetic analysis of PVs.

Virus strain	Accession Number	Nucleotide identities
Bovine papillomavirus type 1 isolate IPV 03 major capsid protein (L1) gene partial cds	KU728470.1	76.3%
Bovine papillomavirus type 1 isolate IPV 02 major capsid protein (L1) gene partial cds	KU728469.1	76.3%
Deltapapillomavirus 4 isolate CRO-5 L1 gene partial cds	JX046509.1	73.2%
Bovine papillomavirus type 2 complete genome	M20219.1	71.8%
Bos taurus papillomavirus 13 strain 14RO12 complete genome	KU519390.1	70.2%
Bovine papillomavirus type 13 strain Hainan complete genome	KM258443.2	70.2%
Deer papillomavirus (DPV) complete genome	M11910.1	59.4%
European elk papillomavirus complete genome	M15953.1	58.9%
Reindeer papillomavirus complete genome	AF443292.1	58.9%
Western roe deer papillomavirus 1 isolate CcPV-1 complete genome	EF680235.1	57.0%
Cervus elaphus papillomavirus 1 strain IT 1127 complete genome	JQ744282.1	56.8%
Bovine papillomavirus type 5 complete genome	AF457465.1	56.3%
Epsilonpapillomavirus 1 isolate BRA/05AC12 complete genome	KU707883.1	56.1%
Ovine papillomavirus - 1 complete genome	gi 9629249:5635-7128	55.2%
Camelus dromedarius papillomavirus type 2 complete genome	HQ912791.1	53.8%
Rangifer tarandus granti papillomavirus isolate VS700028 L1 protein gene complete cds	KJ719447.1	53.1%
Cottontail rabbit papillomavirus complete genome	gi 9627196:5828-7345	50%
Papillomavirus sylvilagi L1 protein gene complete cds	U91411.1	49.8%
Human papillomavirus type 1a L1 gene	AO9292.1	48.8%
Equine papillomavirus 2 complete genome	gi 224460777:5673-71272	44.8%
Equine papillomavirus type 6 complete genome	gb JQ965698.1: 5329-6822	42.7%
Equine papillomavirus 3 complete genome	gi 386645020:5290-6786	40.1%

illomavirus, deer papillomavirus, western roe deer papillomavirus 1 isolate CcPV-1, and *Rangifer tarandus granti* papillomavirus isolate VS700028 showed identities of 58.9%, 58.9%, 59.4%, 57.0% and 53.1%, respectively (Table 2).

Immunofluorescence (IF) staining of ED cells using BPV-1 E2-specific monoclonal antibody yielded a strong signal, while NBL-6 cells scored negative, thus testifying for the specificity of the detection system and reliability of results (Figure 2).

DISCUSSION

Screening of various cell lines and sera for papillomavirus DNA yielded a positive result in case of a cell line derived from equine dermis. The DNA sequence of the obtained amplification product, termed TR-BPV-ED, was genetically more closely related to BPV types than to equine papillomavirus types. The IF staining results were in agreement with this finding, yielding a strong signal when using a BPV-1 E2-specific antibody. Sera used in culture media passage were also tested by PV consensus PCR for papillomavirus contamination but scored consistently negative.

Due to difficulties related to virus isolation, it became necessary to frequently assess cell cultures for viral contaminations. In a previous experiment, all cell lines had tested negative for pestivirus contamination. It is known that pestiviruses generally replicate without inducing a cytopathogenic effect (CPE); they are transmitted by fetal calf sera and interfere with replication of other viruses in cell culture (Merten, 2002). Therefore, cell lines were also tested for contamination

by other viruses including papillomaviruses.

A total of 27 cell lines derived from eight different species and 22 different tissues were examined by PCR using PV consensus primers that amplify a region within the *L1* major capsid gene. Only the ED cell line tested positive in this study. Phylogenetically, the DNA sequence of the obtained PCR product, TR-BPV-ED, was compared to papillomavirus sequences deposited in the GenBank database. Sequence alignment revealed a 40.1–76.3% nucleotide identity with other papillomavirus types from homologous and heterologous hosts. Comparison with BPV-1 sequences originating from cattle and horses showed that TR-BPV-ED was 73.2–76.3% identical to other BPV-1 variants. The isolate in the present study was located on the same branch with *Deltapapillomaviruses* BPV-1, BPV-2, BPV-13, and BPV-14 on the phylogenetic tree, whereas with other papillomavirus types derived from humans and other mammals, i.e. rabbit, camel, deer, elk, horse, sheep, and cattle, PV types were located on distinct genetic branches. BPV infection was detected in an in vitro system where numerous cell culture passages had already been performed. This may explain the genetic aberrations of TR-BPV-ED in comparison to its closest relative, BPV1. On the other hand, DNA viruses are genetically stable viruses. Therefore, it can rather be assumed that an L1 region was amplified corresponding to a yet unknown BPV type. Future whole-genome analysis will help to clarify this issue and provide a better understanding of the genetic diversity of TR-BPV-ED isolates detected from the ED cell line.

Papillomaviruses may establish a persistent infection under the influence of endogenous and environ-

mental factors, and this may increase the spread of viruses by cattle to the same and, in case of Delta-BPVs, other ungulate species (Gil da Costa et al., 2017). Although there are reports on the detection of BPV types 1 and 2 from bovine skin, blood, milk and semen (Santos et al., 2016) in horses, the vast majority of published reports provide evidence that BPV1/2 infection in equids is exclusively associated with sarcoid disease (Nasir and Reid, 2006; Nasir and Campo, 2008; Otten et al., 1993). In one study, it has been reported that sarcoid tumors in cats may be associated with *Deltapapillomavirus* 14 (BPV-14) infection, another example of the broader host spectrum of Delta-BPVs (Munday et al., 2015). Herein, the described papillomavirus sequence was detected from an ED cell line, thus opening the possibility that infection had already been present in the horse from which the cells were explanted (Chambers et al., 2003). Since the ED cell line was provided by collaboration partners many years ago, the exact source of infection/contamination could not be identified. There are some factors that may explain this contamination in ED cell culture. First, horses can be naturally infected by putatively new Delta-BPV, and contaminated ED cell cultures might have been obtained from these naturally infected equids. Therefore, BPV DNA could have been in the ED cell culture from the very beginning. The results of immunofluorescence staining also support that the virus expresses its proteins, at least the early ones. In equine sarcoids, BPV-1 DNA resides in infected cells in an episomal form. In addition, infection may be productive, at least at some stages of disease. There is evidence of the presence of L1 protein and virion assembly in the upper epidermal layer (Bogaert et al., 2010; Brandt et al., 2011). Moreover, Wilson et al. (2013) have visualized BPV-1 virions

in sarcoid tissue by electron microscopy. The ED cell line used in this study is unlikely to support productive infection as it does not provide the proper cellular environment for virion assembly (Doorbar, 2005).

To date, many different PVs have been shown in the skin and mucous membranes of humans and animals. Given the fact that more than two hundred human papillomavirus types are known today, it is more than likely that many novel BPV types will be discovered in the near future. The putative BPV DNA detected in this study is most similar to BPV-1 DNA, with a difference of 24.7%. These results suggest that the obtained viral DNA may represent a putative new type.

Secondly, bovine sera previously used by cooperating researchers might have been contaminated with BPV. Finally, the contamination could have emerged due to laboratory contamination, as animal papillomavirus plasmid vector systems are often used to study molecular and cell biology (Gil da Costa et al., 2017). During these studies, BPV-derived plasmids might have contaminated ED cell cultures, which are frequently exchanged among laboratories, and hence, it is possible that the authors came into possession of such cultures.

CONCLUSION

According to immunofluorescence and sequencing results, viral contamination found in ED cell line could either stem from 1. the animal, from which the cells were initially explanted, 2. from contaminated fetal bovine serum, or 3. from BPV-derived plasmid vectors before the cell line was provided. Therefore, according to the findings of this study, it is highly

Percent Identity																									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23		
1	████	44.8	40.1	42.7	48.8	50.0	49.8	55.2	58.9	58.9	56.8	59.4	57.0	56.3	56.1	53.1	53.8	76.3	76.3	73.2	71.8	70.2	70.2	1	TR-BPV-type-1
2	95.2	████	57.3	58.0	54.0	59.2	59.2	54.2	57.0	55.9	54.7	56.8	54.7	55.6	55.2	53.5	53.3	51.4	51.4	50.2	54.7	53.3	53.3	2	5673-7172
3	109.1	58.9	████	73.5	49.5	52.1	52.1	54.5	51.9	54.2	50.2	55.2	50.7	53.1	53.5	54.7	49.8	46.2	46.2	45.3	49.5	49.3	49.3	3	5290-6786
4	100.6	60.4	30.8	████	51.9	55.2	54.9	52.6	53.8	55.2	53.3	54.0	52.1	55.6	54.9	56.6	54.7	49.3	49.5	48.4	52.8	52.6	52.6	4	JQ965698.1
5	82.1	69.0	78.4	74.1	████	63.1	63.4	56.6	54.9	56.8	57.3	57.3	57.3	58.5	58.7	59.2	57.7	53.1	53.3	50.9	58.2	58.0	58.0	5	A09292.1
6	78.3	58.6	73.2	68.0	49.6	████	99.1	57.5	57.5	56.6	54.5	57.7	53.3	58.5	58.5	58.2	55.6	55.4	55.4	53.3	59.2	61.0	61.0	6	5828-7345
7	79.0	58.7	73.3	68.7	49.0	0.9	████	57.5	57.7	56.8	54.9	57.3	53.8	58.2	58.0	58.7	55.6	55.2	55.2	53.1	58.9	60.8	60.8	7	U91411.1
8	67.7	68.8	64.2	72.3	64.3	61.0	61.1	████	68.3	70.2	67.8	66.9	68.1	65.5	65.5	60.6	63.1	62.4	62.7	60.8	70.0	68.1	68.1	8	5635-7128
9	58.1	61.4	71.4	69.1	68.5	60.9	60.3	42.4	████	74.9	71.8	73.5	74.6	65.3	65.0	60.6	60.1	65.7	65.7	63.8	71.4	70.0	70.0	9	M15953.1
10	58.5	64.1	64.8	65.5	63.4	63.4	62.7	39.2	31.5	████	73.0	73.7	72.5	65.3	65.5	59.9	62.9	64.3	64.6	63.1	70.9	67.8	67.8	10	AF443292.1
11	63.0	66.8	75.7	70.5	62.5	68.4	67.1	43.4	36.3	34.5	████	71.1	91.8	66.2	66.2	61.5	62.7	64.8	65.0	63.4	70.9	70.2	70.2	11	JQ744282.1
12	57.5	62.2	62.8	68.4	62.2	60.7	61.8	45.0	33.6	33.2	37.4	████	71.6	65.7	66.0	61.7	62.2	67.1	67.4	66.0	69.2	70.0	70.0	12	M11910.1
13	62.6	67.0	74.7	73.6	62.5	71.5	70.1	43.3	31.8	35.4	8.9	36.7	████	66.4	66.4	62.0	63.1	64.3	64.3	63.4	69.2	68.8	68.8	13	EF680235.1
14	63.3	65.2	68.5	65.2	58.8	59.1	59.7	46.6	47.8	47.5	45.3	46.3	45.0	████	99.3	64.8	64.8	62.9	63.1	62.0	66.7	66.4	66.4	14	AF457465.1
15	64.0	66.4	67.1	67.0	58.3	59.1	60.2	46.6	48.3	47.1	45.3	45.9	45.0	0.7	████	64.3	64.8	62.7	62.9	61.7	66.4	66.2	66.2	15	KU707883.1
16	68.2	71.5	65.1	63.6	56.3	60.3	59.3	53.9	53.5	55.7	51.5	51.3	50.5	46.0	46.9	████	61.7	58.5	58.7	56.3	63.4	63.1	63.1	16	KJ719447.1
17	68.3	71.6	79.2	68.8	60.5	66.9	66.9	51.1	57.6	51.7	51.6	52.5	51.1	47.6	47.6	51.9	████	59.6	59.9	58.2	64.8	65.3	65.3	17	HQ912791.1
18	21.2	66.6	78.1	72.3	63.7	57.5	58.0	45.7	39.7	41.8	40.8	36.9	41.9	43.7	44.2	49.9	49.4	████	99.8	94.1	80.5	80.5	80.5	18	KU728470.1
19	21.2	66.7	78.2	71.6	63.2	57.6	58.1	45.3	39.7	41.4	40.4	36.5	42.0	43.3	43.8	49.4	48.9	0.3	████	94.4	80.8	80.8	80.8	19	KU728469.1
20	23.5	66.9	78.0	72.2	66.5	60.1	60.7	46.6	40.8	41.6	41.1	36.6	41.2	43.0	43.5	52.0	49.8	0.3	0.0	████	78.6	78.6	78.6	20	JX046509.1
21	33.2	67.2	77.6	71.7	59.9	57.6	58.1	39.6	37.3	38.1	38.0	40.9	41.1	44.5	45.0	47.8	47.2	15.9	15.6	16.3	████	89.7	89.7	21	M20219.1
22	35.8	70.7	79.1	72.7	60.5	53.5	53.9	42.6	39.6	43.5	39.0	39.5	41.8	44.7	45.2	48.5	46.5	15.8	15.5	16.2	11.3	████	100.0	22	KU519390.1
23	35.8	70.7	79.1	72.7	60.5	53.5	53.9	42.6	39.6	43.5	39.0	39.5	41.8	44.7	45.2	48.5	46.5	15.8	15.5	16.2	11.3	0.0	████	23	KM258443.2

Figure 3. Nucleic acid identities and divergences of TR-BPV-ED and other papillomavirus types.

recommended that cells and fetal sera should be used only after being screened for papillomaviruses, besides non-cytopathogenic viruses (pestivirus), to ensure that the results obtained from cell culture studies are accurate and reliable. In this study, cell cultures were investigated for the presence of bovine papillomavirus as a viral contaminant in cell cultures for the first time. In further studies, the entire genome of the probably new BPV type detected from the ED cells needs to be genetically and biologically analyzed, to uncover its identity and possible pathogenic role in equine and/or bovine disease.

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