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ORIGINAL PAPER

Molecular detection of Papillomavirus and immunohistochemical investigation of p53 gene expressions in bovine papillomas and fibropapillomas

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Abstract

Papilloma and fibropapilloma cases are quite common in cattle breeding, which cause economic losses due to decrease in the production of milk, meat, and also impair the quality of hide. In this study, we aimed to determine viral etiology and investigate p53 expression levels with immunohistochemical methods from a total of 30 cases. The study material was collected between 2013 and 2021 in Kars, Turkey. Paraffin embedded tissues were used for earlier cases in which the freshly specimens could not be provided. Cases were investigated for papillomavirus etiology with polymerase chain reaction (PCR) using FAP59/FAP64 and MY09/MY11 primer pairs. In 20 of the 30 cases papillomaviruses were identified, and 10 cases were identified as Bovine papillomavirus-1 (BPV-1), 1 case as BPV-2, 1 case as BPV-12, and 1 case as equus caballus papillomavirus 2 (EcPV-2) after sequence analysis. p53 immunostaining was also performed, and the cases were graded according to the positively stained cells. In conclusion BPV-12 was detected for the first time in our country, EcPV-2 was detected first time in cattle indicating cross species infection and p53 was staining most evident in BPV-1 and BPV-2 cases and BPV-12 and EcPV-2 was not stained.

Keywords Cattle · Immunohistochemistry · Papilloma · Polymerase chain reaction · p53

Introduction

Bovine Papillomaviruses (BPVs) are non-enveloped viruses with circular double stranded DNA consisting of approximately 8.000 bases. They infect epithelial and mucosal tissues of different cattle and other species (Araldi et al. 2014; Figueirêdo et al. 2020; Meng et al. 2021). Viral replication takes place in the nucleus and progeny virions are released by lysis of the infected cells (Hong and Kim 2015). BPVs are classified in *Papillomaviridae* under genus *Papillomavirus* (Ata et al. 2021). BPVs consist of 29 genotypes and 5 subgenera (Cutarelli et al. 2021). These are *Deltapapillomavirus* (BPV-1, BPV-2, BPV-13, and BPV-14), *Xipapillomavirus* (BPV-3, BPV-4, BPV-6, BPV-9, BPV-10, BPV-11, BPV-12, BPV-15, BPV- 17, BPV-20, BPV-23, BPV-24, BPV-26, BPV-28, and BPV-29), *Epsilon papillomavirus* (BPV-5, BPV-8, and BPV-25), *Dyoxipapillomavirus* (BPV-7) and *Dyokappapapillomavirus* (BPV-16, BPV-18, and BPV 22). BPV-19, 21, and 27 are unclassified (Sauthier et al. 2021). BPVs most of the time are limited to host species but BPV-1, BPV-2, and BPV-13 are reported to infect equids and cause tumors in these species (Shanshol and Ahmed 2021). BPVs induce both malign and benign tumors, such as cutaneous papillomas, fibropapillomas, esophageal, and bladder cancers. Thus, cause major economic losses for meat, milk, and leather quality and production (AL-Salihi et al. 2020; Chagas et al. 2021). Lesions in cattle are seen under age of

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2 and regress within 1 to 14 months without any intervention (Beytut 2017). Most lesions are benign, but depending on various factors such as genetics and environmental conditions they may transform into malign lesions (Dagalp et al. 2017). Primary host of BPVs is cattle and virus infects through skin scratches and other lesions (Tan et al. 2012). Virus can also transmit through contact of milk, semen, and urine, and vertical transmission is also possible (Ata et al. 2018). Malnutrition, hormonal imbalances, long exposure to direct sunlight, and mutations are among other reasons that increase the risk of infection with virus (Timurkan and Alcigir 2017). Diagnosis of BPV is made by clinical inspection, histopathology, and immunohistochemical methods. Polymerase chain reaction (PCR) is also a sensitive and current method for identifying and genotyping of BPVs (Hamad et al. 2017).

There are various reports of different papillomavirus species in Turkey. Most reported BPV genus is *Deltapapillomavirus* (BPV-1 and BPV-2) and BPV-3, BPV-4, BPV-6, BPV-7, BPV-8, BPV-9, BPV-10, BR-UEL6 like, and BAPV-6 are also found in different studies (Tan et al. 2012; Ataseven et al. 2016; Dagalp et al. 2017; Timurkan and Alcigir 2017; Yıldırım et al. 2021). There are also reports of papillomaviruses in goats (Dogan et al. 2018), dogs (Oğuzoğlu et al. 2017), and horses (Kanat et al. 2019).

p53 is an important tumor suppressive protein that regulates cellular mechanisms like apoptosis, arrest of the cell cycle, and senescence which are a result of various factors like oxidative stress, hypoxia, DNA damage (Finlay et al. 2012; AL-Salihi et al. 2020). Apoptosis is a process that balances ratio of cell proliferation to cell death, regulates growth, and homeostasis. Impairment of this process is one of the characteristic features of cancer development (Bocanetti et al. 2015). Tumor development in human papillomavirus (HPV) is induced by ubiquitin dependent inactivation of p53 by viral oncogenic protein E6. This leads to impairment of cell cycle control, cell differentiation, increase in mutations, and chromosomal instability (Mantovani and Banks 1999; Ilves et al. 2003; Araldi et al. 2014; Thaiwong et al. 2018).

This study aims to evaluate p53 levels with immunohistochemistry in papilloma and fibropapilloma cases which are characterized by molecular methods as BPV-1, BPV-2, BPV-12, and equus caballus papillomavirus type 2 (EcPV-2).

Materials and methods

Tissue samples

The materials of this study consist of tissues from papilloma and fibropapilloma cases which belong to 30 cattle taken from different areas between years 2013and 2021 in Kars, Turkey. Detailed information about material is given in Table 1. Six nontumorous cattle skin samples are used as negative control.

For molecular investigation paraffin embedded tissues were used for cases that freshly takes samples were not available.

Nucleic acid extraction from freshly obtained samples

Biopsy samples were homogenized with gloved hands in a class 2 safety cabinet in a sterile petri dish and cut into several pieces using sterile scalpel. Then, shredded tissue is transferred to 1.5 ml polystyrene tubes and 900 µl phosphate buffered saline (PBS) was added. After vortexing for 2 min vigorously, they were centrifuged at 3000 rpm for 15 min. Supernatant was taken and DNA extraction was performed using phenol/chloroform described previously (Sambrook and Russell 2001). Extracts were stored at − 20 °C until further processing.

Nucleic acid extraction from paraffin embedded tissue blocks

Procedure was performed as described previously by Pikor et al. (2011). The procedure starts with deparaffinization with xylene, rehydration with ethanol baths containing 100%, 70%, and 50%.In addition, tissues are treated with lysis buffer containing proteinase K in 55 °C until tissues are fully dissolved. Viral nucleic is precipitated with isopropyl alcohol. Extracts were stored at − 20 °C until further processing.

Molecular analysis

PCR was used for investigation of papillomavirus nucleic acid. L1 ORF (Open Reading Frame) was chosen because it is conservative. Primer pair FAP59/FAP64 (Forslund et al. 1999) and MY09/MY11 (Ogawa et al. 2004) primer pairs were used as described, expected amplicon sizes were 478 bp and 450 bp, respectively. PCR products were visualized in a transilluminator after electrophoresis in 1% agarose gel containing Safe-Red (Safe ViewTM Cat No: G108-R, Canada) DNA stain.

Phylogenetic analysis

Amplicons having sufficient DNA yield and were suitable for sequence analysis were sent to a commercial company (BM Yazilim Danis. ve Lab. Sis. Ltd. Sti, Ankara) for sanger sequencing. Sequence assembly and editing were done with Bioedit (Version 7.0.5.3) and Clustral W (Hall 1999). Sequence similarities were compared with the GenBank

Table 1 Information about animals

Case no	Age (month)	Sex	Breed	Localization	Diameter (cm)	Macroscopy	Histopathology
1	36	Male	Brown Swiss	Hoof	2	Cauliflower-like	Fibropapilloma
2	18	Male	Holstein	Penis	3	Cauliflower-like	Fibropapilloma
3	24	Male	Holstein	Penis	3	Cauliflower-like	Fibropapilloma
4	12	Female	Simmental	Hoof	1	Cauliflower-like	Squamous Papilloma
5	12	Male	Simmental	Penis	2	Cauliflower-like	Fibropapilloma
6	12	Male	Holstein	Neck	4.5	Cauliflower-like	Squamous Papilloma
7	60	Male	Brown Swiss	Hoof	3	Cauliflower-like	Fibropapilloma
8	72	Female	Brown Swiss	Teat	7	Nodular	Fibropapilloma
9	24	Female	Simmental	Teat	6	Cauliflower-like	Squamous Papilloma
10	12	Male	Holstein	Hoof	1	Nodular	Squamous Papilloma
11	10	Male	Simmental	Penis	1.5	Cauliflower-like	Fibropapilloma
12	24	Male	Brown Swiss	Penis	6	Cauliflower-like	Fibropapilloma
13	12	Male	Simmental	Penis	3.5	Nodular	Fibropapilloma
14	24	Female	Simmental	Abdomen	9.5	Cauliflower-like	Squamous Papilloma
15	9	Female	Simmental	Abdomen	7	Cauliflower-like	Squamous Papilloma
16	9	Female	Holstein	Abdomen	5	Cauliflower-like	Squamous Papilloma
17	48	Female	Simmental	Teat	1	Cauliflower-like	Squamous Papilloma
18	24	Female	Holstein	Teat	1	Cauliflower-like	Fibropapilloma
19	12	Female	Simmental	Abdomen	4	Cauliflower-like	Squamous Papillom
20	12	Female	Brown Swiss	Neck	5	Cauliflower-like	Squamous Papillom
21	24	Male	Simmental	Penis	3	Nodular	Fibropapilloma
22	24	Male	Simmental	Penis	3.5	Cauliflower-like	Fibropapilloma
23	9	Male	Brown Swiss	Penis	3	Nodular	Fibropapilloma
24	12	Male	Brown Swiss	Penis	4	Nodular	Fibropapilloma
25	24	Male	Brown Swiss	Penis	5	Nodular	Fibropapilloma
26	48	Male	Holstein	Hoof	3	Nodular	Squamous Papilloma
27	24	Female	Simmental	Teat	5	Nodular	Squamous Papilloma
28	24	Female	Holstein	Eye Area	4	Cauliflower-like	Squamous Papilloma
29	12	Female	Simmental	Mandibular Area	1	Cauliflower-like	Squamous Papilloma
30	24	Female	Simmental	Teat	6	Nodular	Squamous Papilloma

nucleotide sequence database using the Basic Length Alignment Search Tool (BLAST) software of the National Center for Biotechnology Information (NCBI) (Altschul et al. 1997). Phylogenetic analysis of gene sequences was performed using MEGA7 software (Tamura et al. 2011). Neighbor-joining method of software was selected, and the sequence differences were calculated with the Kimura two-parameter model, confidence level was assessed by bootstrapping using 1000 replicates.

Histopathology

Tumor masses were photographed before and after excision. Tissues were fixed in 10% buffered formaldehyde (Merck). After following the routine procedure, sections of 5 µm for Hematoxylin & Eosin (H&E) staining and sections of 4 µm for immunohistochemical staining were taken and placed onto poly-L-lysin coated slides.

For observing histopathological changes H&E staining was used, slides were examined thoroughly under light microscope (Olympus Bx53) by at least two pathologists. Photographs were taken using Cell ^P software (Olympus Soft Imaging Solutions GmbH, 3,4). Detailed analysis of taken photographs were made with Image J software (1.51j8).

Masson trichrome aniline blue staining

Staining was performed as described by the manufacturer (Facepath, Barcode 8681065132824).

Immunohistochemistry

The routine streptavidin–biotin peroxidase complex method was used according to the manual instructions of kit (Thermo Scientific Histostain-Plus IHC Kit, HRP, broad

spectrum, REF: TP-125-HL). Primary antibodies were used after antigen retrieval (the sections were boiled in Citrate Buffer Solution (pH 6) for 25 min in the microwave oven at 800 W) and nonspecific protein blocking. Details of primer antibodies used in this study are given in Table 2. The reactions were detected with 3,3′-Diaminobenzidine (DAB) chromogen. Counter stainings were conducted using hematoxylin. Then, the glass slides were mounted with Entellan and coated with immune mount. For control sections, instead of the primary antibody, PBS was applied in drops on the sections.

Immunohistochemical analysis of BPV and p53 expressions was evaluated using a grading system according to positively stained cell number on sites that has intensive staining characteristics. 3 different areas for each slide were examined at 40 × magnification. Grading was determined as following (−) no immunoreactivity; (+) low, 1–10% positivity in cell of the area; (++) moderate, 11–59% positivity in cell of the area; and (+++) intense, higher than 60% positivity in cell of the area (Beytut 2017).

Results

Molecular results

In total, 20 out of 30 samples had correct sized amplicons using FAP59/FAP64 and/or MY09/MY11 primer sets. From positive samples, not all amplicons sufficient and good quality DNA quantity, 13 of the 20 positive amplicons were decided to be suitable for Sanger sequencing. Two independent phylogenetic trees were constructed with sequences obtained using FAP59/FAP64 ($n = 2$) and MY09/MY11 ($n = 11$) primers. After the sequencing, 13 samples were classified as BPV ($n = 10$ BPV-1, n = 1 BPV-2, $n = 1$ BPV-12) DNA, 1 sample was classified as EcPV (EqPV-2). Sequences were deposited to GenBank.

The GenBank accession numbers are OK157504, OK157505, OK157507, OK157508, OK157509, OK157510, OK157511, OK157512, OK157513, OK157514, OM362622 with MY09/11 primers sequences and OK377064, OM202575 with FAP59/64 primers sequences.

The constructed phylogenetic trees based on the sequences amplified with FAP59/FAP64 and MY09/MY11 with the GenBank reference strains are shown in Figs. 1 and 2 respectively. Nucleotide homologies of each sample and the respective reference strains were also checked.

Partial BPV sequences provided with MY09/MY11 primer pair in this study were similar 98.8–100% among themselves and other BPV-1 references from GenBank at nucleotide level. In addition, partial sequence of EcPV-2 isolated from a cattle penis was 99.2% similar to EcPV-2 (MT063186). Similarities were between 55.5% and 55.7% when BPV and EcPV-2 sequences of the study were compared. Partial sequence of BPV-2 produced with FAP59/FAP64 pair was similar 99.3% to a reference BPV-2 (KF284153) obtained from GenBank. When partial sequence of BPV-12 (produced with FAP59/FAP64 primer pair) analyzed it was 97.4% similar to a BPV-12 (JX431294) reference strain from GenBank.

Gross findings

Cattle were of under age 2 (average 1.92); of which 16 male (53.33%) and 14 female (46.66%). Breed of cattle included were Simmental (14/30–46.66%), Brown Swiss (8/30–26.66%) and Holstein (8/30–26.66%). Localizations of samples were hoof (5/30—16.66%), penis (11/30—36.66%), neck (2/30–6.66%), teat (6/30–19.99%), abdomen (4/30–13.33%), eye area (1/30–3.33%) and mandibular area (1/30–3.33%). Some were exophytic cauliflower like cutaneous lesions (20/30–66.66%), some were nodular (10/30–33.33%) with or without peduncles. Masses were various in diameter (1–10 cm, average of 3.78 cm), solitary or multiple and oval shape. Masses that were grayish-white had relatively firm consistency. In cases that were identified as fibropapillomas, had rough grayish-white surface due to connective tissue proliferation. Some masses had hemorrhagic and ulcerative surfaces and color was brownish black because of these complications (Fig. 3A–F).

Microscopic findings

8 out of the 10 BPV-1 positive cases were identified as squamous papilloma and 2 were identified as fibropapilloma. Also, BPV-2 was identified as fibropapilloma. BPV-12 was identified as papilloma and EcPV-2 as fibropapilloma in histopathological examinations. In squamous papillomas, stratum corneum had average or severe orthokeratotic or parakeratotic hyperkeratosis and in stratum spinosum acanthosis (characterized by hyperplasia of the cells of this layer) and rete ridges (epithelial extensions that project from epidermis to dermis) was observed. In addition to these lesions severe hydropic and

Table 2 Information on primary antibodies used for immunohistochemical evaluations

Primary antibodies	Company and Catalog numbers	Dilution	Incubation condition
Mouse monoclonal BPV antibody	MyBioSource, MBS320197	1/100	Over night, 4 °C
Mouse monoclonal p53 antibody	MyBioSource, MBS438209	1/100	Over night, 4 °C

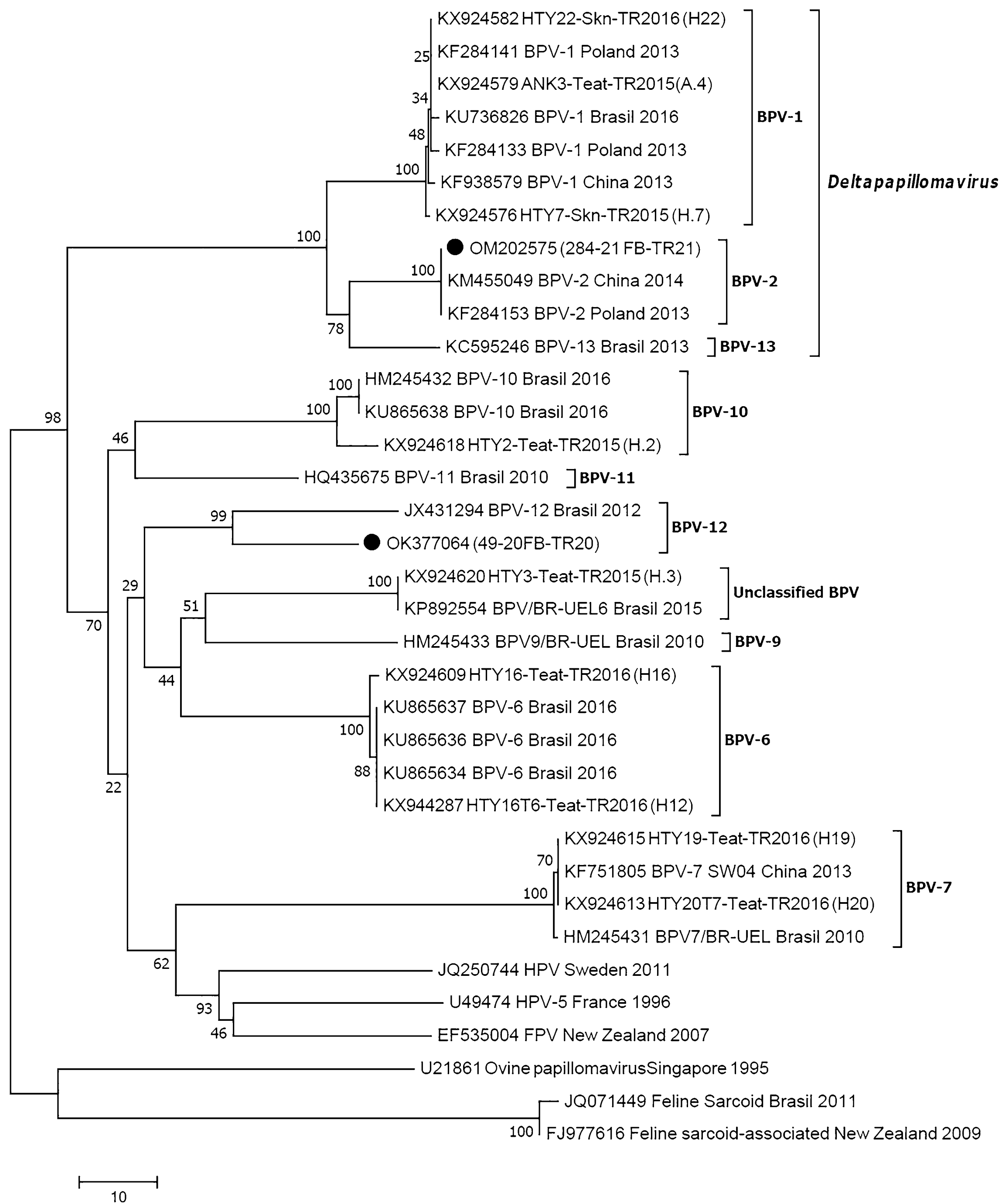

Fig. 1 Phylogenetic tree according to FAP59/FAP64 primer pair

vacuolar degeneration in keratinocytes in stratum spinosum, increase of keratohyalin granules in keratinocytes located in stratum granulosum and stratum spinosum. In addition in the same layers, koilocytes was detected which had clear eosinophilic cytoplasm and picnotic nucleus located eccentrically. Intranuclear eosinophilic inclusion bodies in keratinocytes was noteworthy. Cells of epidermal layer was well differentiated and a few mitotic figures were other among important histopathological findings. Fibropapilloma cases had finger like projections with dense connective tissue bundles consisting of collagen fibers and fibroblasts between them, in addition to other histopathological changes. In dermal layer, intense vasculitis, neutrophil leukocyte infiltration, bacterial colonies, hemorrhage, and ulcerative regions were observed (Fig. 4A–E).

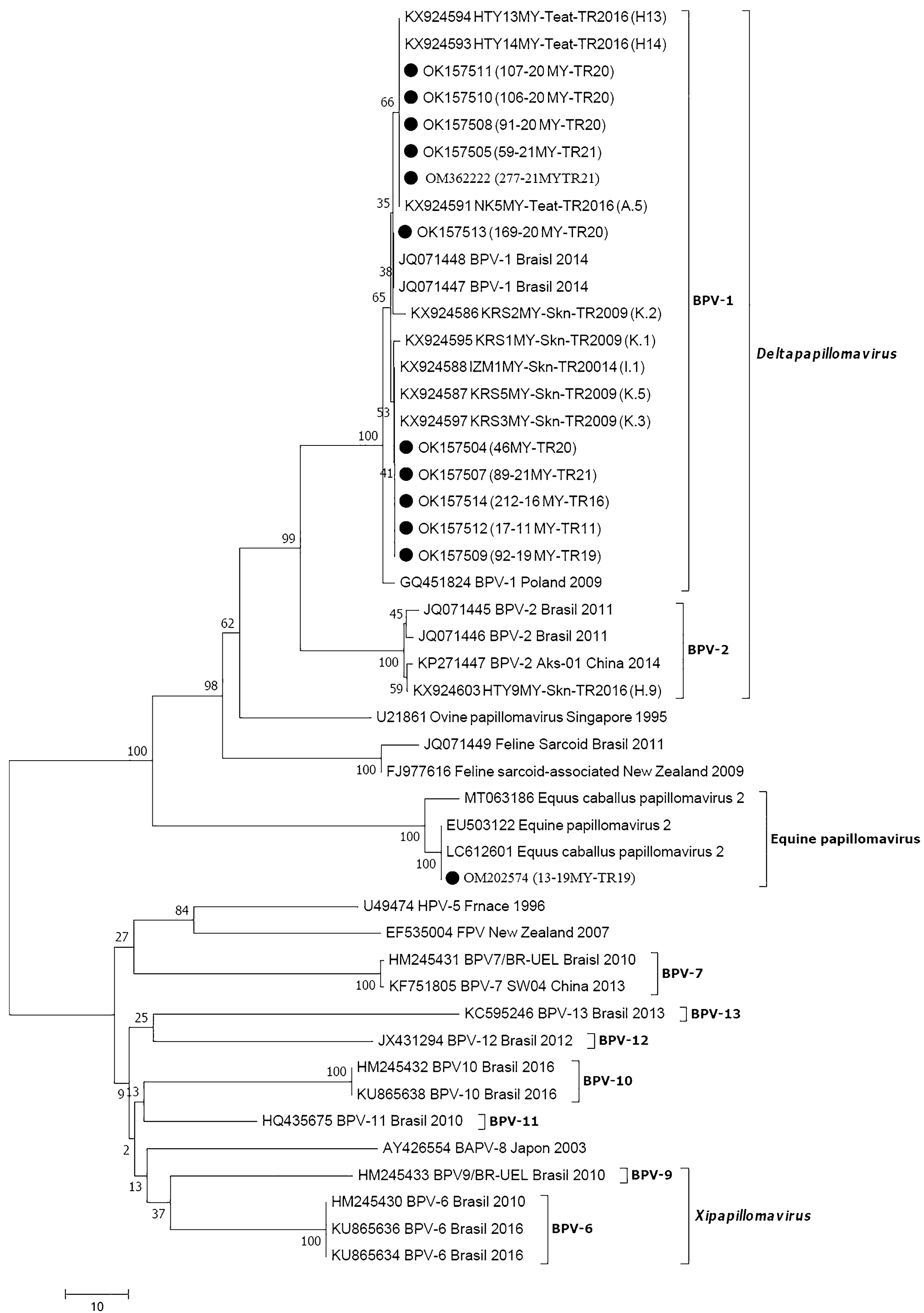

Fig. 2 Phylogenetic tree according to MY09/MY11 primer pair

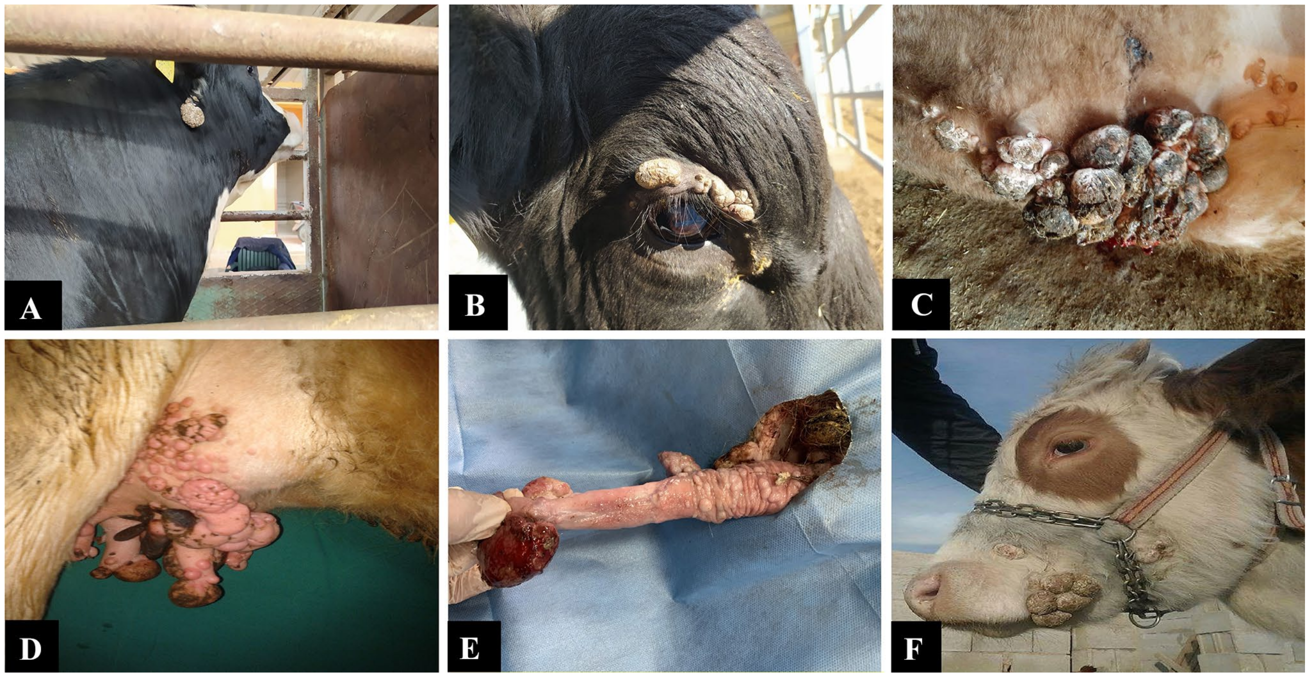

Fig. 3 Tumoral masses exhibiting cauliflower-like and nodular growths from **A** Neck. **B** Eye area. **C** Abdomen. **D** Teat. **E** Penis **F** Mandibular area

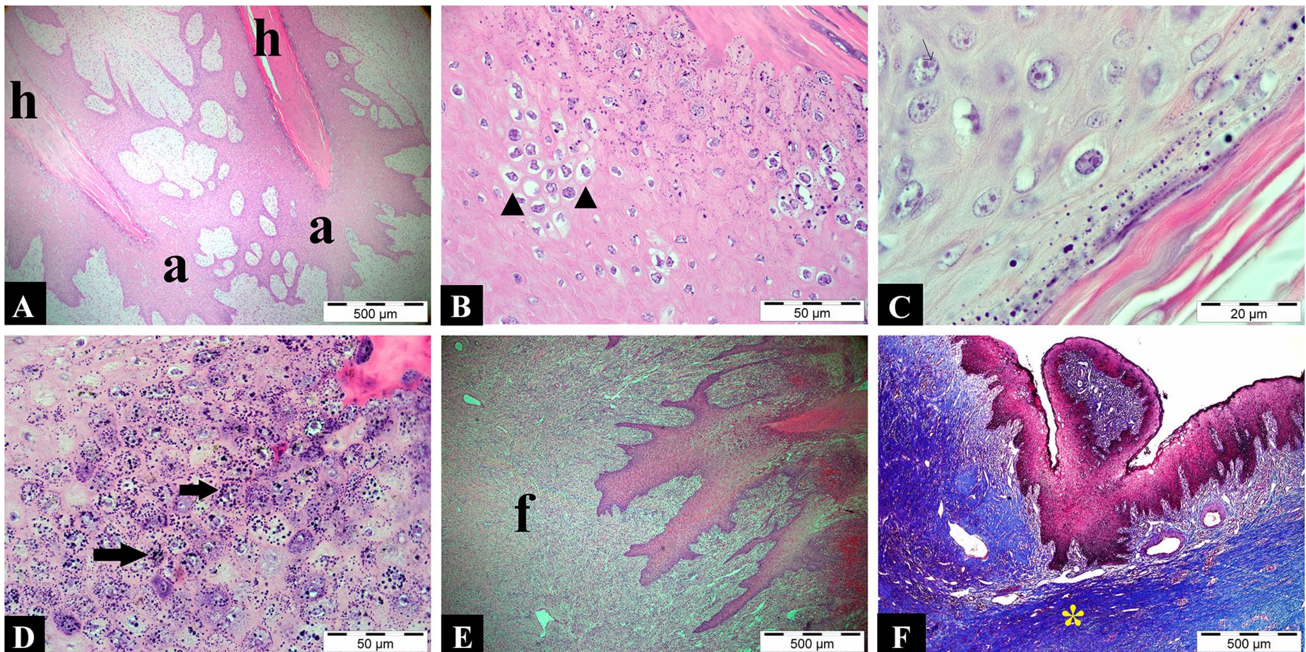

Fig. 4 **A** Hyperkeratosis (h), acanthosis (a), (H&E, Bar: 500 µm). **B** Hydropic degeneration of keratinocytes (arrowheads), (H&E, Bar: 50 µm). **C** Intranuclear eosinophilic inclusion bodies (thin arrow), (H&E, Bar: 20 µm). **D** Keratohyalin granules (arrows), (H&E, Bar: 50 µm). **E** Dense bundles of connective tissue (f) extending in different directions between finger-like projections (H&E, Bar: 500 µm). **F** Masson Trichrome Aniline Blue Staining, connective tissue growths in the dermis (star), (Bar = 500 µm)

Masson trichrome Anilin blue staining findings

Intense collagen deposition was observed in fibropapilloma cases. In rete pegs and regions of dermis neighboring epidermis, increased fibroblastic activity was observed. Fibroblastic activity was seemingly decreased towards center of the masses (Fig. 4F).

Immunohistochemical findings

Scores of immunopositivity according to distinct BPV types and staining patterns are given in Table 3. Negative control group was not stained with BPV or p53. In papilloma and fibropapilloma cases, intranuclear dark brown BPV expressions in keratinocytes of granular layer of epidermis was detected. There was no staining of connective tissue (fibrocyte, fibroblast, collagen fibers) in fibropapillomas, (Fig. 5A, B). p53 immunopositivity was detected in 25 of the 30 cases (83.33%). Keratinocytes of stratum corneum, granulosum, and spinosum had weak positive reaction. Positive reactions were most obviously seen in parabasal and basal tumor cells of rete ridges (yellow-brownish coloring in the nucleus of these cells). There was no staining of p53 in dermal layer (Fig. 6A, B).

Discussion

Deltapapillomaviruses consist of highly pathogenic BPVs like BPV-1, BPV-2, BPV-13, and BPV-14 (Cutarelli et al. 2021). *Deltapapillomaviruses* infect both epidermis and dermis so they can cause epithelial papillomas or cutaneous fibropapillomas (Bertagnolli et al. 2020; Bianchi et al. 2020). BPV-1 and BPV-2 are also associated with bladder cancer of the cattle fed with bracken fern; alimentary tract, anogenital, teat, and penis fibropapillomas (Grindatto et al. 2015; Ataseven et al. 2016; Yamashita-Kawanishi et al. 2019). BPV-1 and BPV-2 can also infect other species,

Table 3 Immunpositivity scores and staining patterns according to different BPV types

Case No	BPV	Pattern	p53	Pattern	PCR	Type
1	+++	Nuclear	+	Nuclear	MY positive	BPV-1
2	+++	Nuclear	+	Nuclear	Negative	No sequence
3	+++	Nuclear	+	Nuclear	Negative	No sequence
4	+++	Nuclear	+	Nuclear	Negative	No sequence
5	+++	Nuclear	–	Negative	MY positive	EcPV-2
6	+++	Nuclear	+++	Nuclear	MY positive	BPV-1
7	+++	Nuclear	+	Nuclear	Negative	No sequence
8	+++	Nuclear	+++	Nuclear	Negative	No sequence
9	+++	Nuclear	+++	Nuclear	MY positive	No sequence
10	++	Nuclear	+	Nuclear	Negative	No sequence
11	++	Nuclear	+	Nuclear	MY positive	No sequence
12	+	Nuclear	+	Nuclear	MY positive	No sequence
13	+	Nuclear	–	Negative	FAP positive	No sequence
14	+++	Nuclear	+++	Nuclear	MY positive	BPV-1
15	+++	Nuclear	+++	Nuclear	MY positive	BPV-1
16	+++	Nuclear	++	Nuclear	MY positive	BPV-1
17	+++	Nuclear	+++	Nuclear	MY positive	BPV-1
18	+++	Nuclear	+++	Nuclear	MY positive	No sequence
19	+++	Nuclear	++	Nuclear	MY positive	BPV-1
20	++	Nuclear	++	Nuclear	MY positive	BPV-1
21	+	Nuclear	–	Negative	MY positive	No sequence
22	++	Nuclear	+	Nuclear	Negative	No sequence
23	+	Nuclear	+	Nuclear	MY positive	BPV-1
24	+++	Nuclear	+	Nuclear	FAP positive	BPV-2
25	+++	Nuclear	+	Nuclear	Negative	No sequence
26	+++	Nuclear	+	Nuclear	Negative	No sequence
27	+++	Nuclear	+	Nuclear	MY positive	No sequence
28	+	Nuclear	–	Negative	MY positive	BPV-1
29	+	Nuclear	–	Negative	FAP positive	BPV-12
30	+	Nuclear	++	Nuclear	Negative	No sequence

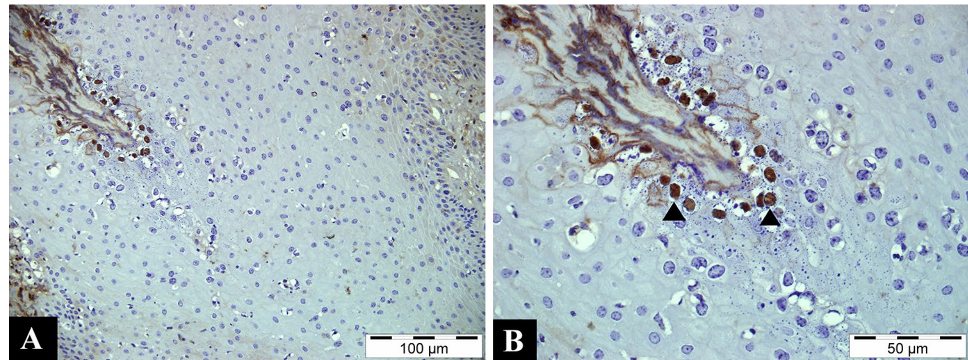

Fig. 5 **A** BPV immune positive reactions in the nuclei of cells in the stratum granulosum layer of the epidermis, (IHC, Bar: 100 µm). **B** Higher magnification, intranuclear BPV expressions (arrowheads), (IHC, Bar: 50 µm)

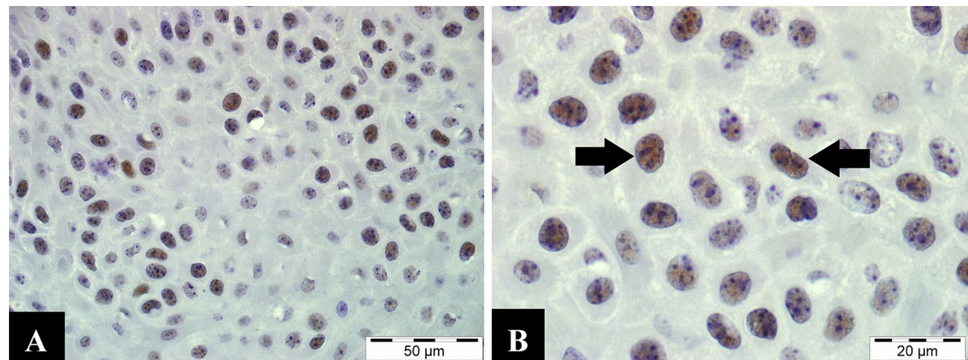

Fig. 6 A Intranuclear p53 positive reactions in tumoral cells at the periphery of rete peg structures, (IHC, Bar: 50 µm). **B** Higher magnification, yellow-brownish p53 expression in the nuclei of tumoral cells (arrows), (IHC, Bar: 20 µm)

such as domestic cats, zebras, giraffes, and sable antelopes and may cause different types of tumors like equine sarcoids. *Deltapapillomaviruses* can be isolated from peripheral blood of apparently healthy animals without any skin lesions (Kanat et al. 2019; Chagas et al. 2021; Cutarelli et al. 2021). Hamad et al. (2017) reported that warts of cattle can be located in many regions of the body, but teat and neck are the most affected areas. As reported in the previous studies, this study identified BPV-1 and BPV-2 from papilloma and fibropapilloma cases of hoof (Dagalp et al. 2017), abdomen (Peng et al. 2019), teat (Chagas et al. 2021), eye area (Pangty et al. 2010), neck (Hong and Kim 2015), and penis (Bertagnolli et al. 2020).

Xipapillomaviruses (BPV-3, BPV-4, BPV-6, BPV-9, BPV-10, BPV-11, BPV-12, BPV-15, BPV-17, BPV-20, BPV-23, BPV-24, BPV-26, BPV-28, and BPV-29) are only epitheliotropic and cause only papillomas (Hong and Kim 2015; Yamashita-Kawanishi et al. 2020; Sauthier et al. 2021). Chagas et al. (2021) evaluated papillomatous lesions of neck and teat from cattle and identified BPV-12 as the causative agent. In other studies, Sauthier et al. (2021) and Bianchi et al. (2020), researchers identified BPV-12 from teat papillomas. Similarly, Savini et al. (2016) identified BPV-12 from a tumor taken from facial area of a cattle. In this study BPV-12 was identified from a mass taken from mandibular area similar to other studies (Araldi et al. 2014; Hong and Kim 2015). The tumor was diagnosed as papilloma with microscopic examination.

The most common BPV genus reported in Turkey by researchers are *Deltapapillomavirus*, mostly BPV-1 and BPV-2. Other genotypes are also reported such as BPV-3, BPV-4, BPV-6, BPV-7, BPV-8, BPV-9, BPV-10, BR-UEL6 like and BAPV-6 (Tan et al. 2012; Ataseven et al. 2016; Dagalp et al. 2017; Timurkan and Alcigir 2017; Yıldırım et al. 2021). There has also been reports on BPV-1 and BPV-2 crossing host species barrier and causing infection in horses (Kanat et al. 2019). As far as is known there is no study reporting BPV-12, so this is the first study to report BPV-12 presence in Turkey.

There are nine equus caballus papillomaviruses (EcPV 1–9) and two equus asinus papillomavirus (EaPV 1–2) genotypes that has the potential to cause benign or malign proliferative lesions or more malign cancers in horses. Papillomaviruses are known to cross host species barrier and infect other species. Most common example is isolation of BPV-1, BPV-2 and BPV-13 from equine sarcoids. Likewise, BPV-14 was isolated from feline sarcoids and ovis aries papillomavirus type 2 (OaPV2) has been reported to cause lesions like sarcoids in pigs (Jones 2021; Munday et al. 2021). These occurrences indicate the infective potential of papillomaviruses between different species. Even though studies report BPVs infecting horses, as far as is known there is no report

of equine papillomavirus transmission to cattle. This study reports EcPV-2 presence in a bovine penis for the first time. EcPV-2 is also reported in genital lesion of horses (Jones 2021). In the region where study is conducted cattle and horses are in close proximity especially in pastures. Papillomaviruses spread through direct contact or via fomites (Tan et al. 2012; Timurkan and Alcigir 2017; Ata et al. 2018; Jones 2021). Papillomaviruses are resistant to cold and desiccation and they do not have an envelope, for these reasons they are not easily affected by environmental factors. In a contaminated pasture virions can easily infect an animal with skin abrasions. Moreover, mechanical vectors like black flies are thought to have a role in carrying the virions (Jones 2021). These conditions are consistent with the current state of husbandry in the region of the study. Specific information is not available in first anamnesis and although authors tried to contact breeders no further information could be obtained. However, the authors think detection of EcPV-2 from cattle is because of close contact from cattle and horses.

P53 protein has a crucial role in cell cycle mechanisms. Lack of p53 mediated suppression can lead to development of squamous cell carcinoma (SCC), ameloblastoma in humans and BPV induced tumors in cattle (Bocanetti et al. 2015; AL-Salihi et al. 2020). *Deltapapillomaviruses* are common in cattle and they contain a complete set of oncogenes. For this reason, they are associated with malign cell transformation and they are known as high risk BPVs (Sauthier et al. 2021). There is little data on how BPV oncogenes affect and impair cell pathways and lead to neoplastic transformations (AL-Salihi et al. 2020). Genomic instability occurs because of expression the E6 and E7 oncogenes. E6 oncoprotein is responsible for impairment of actin cell skeleton and clastogenicity. It affects DNA repair system with ubiquination of p53, cell proliferation and proteosome degradation (Mantovani and Banks 1999; Ilves et al. 2003; Araldi et al. 2014). Scobie et al. (1997) investigated E6 protein of BPV-4 in an in vitro study and concluded that it had other functions in cell transformation independent of p53. Increase in p53 expressions has been investigated in horse (Finlay et al. 2012; Kanat et al. 2019), dog (Thaiwong et al. 2018) and bovine papillomas (Bocanetti et al. 2015; AL-Salihi et al. 2020; Hassanien et al. 2021). Finlay et al. (2012) researched BPV-1 isolated from equine sarcoids in two cell lines and found the overexpression of p53 is cytoplasmic. Kanat et al. (2019) demonstrated expression of p53 in two equine sarcoid cases caused by BPV-1. They also found no p53 staining of in equine sarcoids caused by EcPV-2. Similarly in this study there was no p53 staining of EcPV-2 in cattle tissue. Thaiwong et al. (2018) found intensive immunopositivity for p53 in benign papillomatous lesions of dogs. In addition, they reported invasive SCCs originated from benign viral papillomas had more intense p53 staining. Bocanetti et al. (2015) observed cytoplasmic and perinuclear p53 positive reactions in basal and parabasal epithelial cells of bovine fibropapillomas. They suggested apoptosis regulating proteins like Bcl-2 and p53 has an important role in development of cutaneous fibropapillomas. AL-Salihi et al. (2020) reported cytoplasmic and perinuclear p53 positivity in stratum corneum, parabasal and basal layers in papilloma and fibropapilloma cases. They suggested these p53 accumulation in cell cytoplasm is due to some important pathways and these pathways impair the tumor suppression function of p53 protein (Finlay et al. 2012; AL-Salihi et al. 2020). Hassanein et al. (2021) identified BPV-2 from face, head, neck, scapula and hind limb areas of cattle and considered the impaired p53 function induced by BPVs neoplastic transformation mechanism as the cause of the increased of p53 expression. Our findings were in line with the studies (Bocanetti et al. 2015; AL-Salihi et al. 2020; Hassanein et al. 2021) in this regard, weak p53 immunoreactivity in keratinocytes of stratum corneum, granulosum and spinosum layers and more intense positivity in basal and parabasal tumor cells of the rete ridges. Differently from EcPV-2, p53 positivity of ten BPV-1 and one BPV-2 was quite evident. There was no staining of p53 in BPV-12 case. Nuclear reactions were more prominent than cytoplasmic reactions. The rate of immunopositivity of p53 was 83.33% in overall cases of papilloma and fibropapillomas. This indicates that the p53 has an important role in development of tumors and is a useful marker in diagnosis.

Papillomaviruses are very important for the leather industry within the livestock sector in cattle. In addition, it is important both in terms of racing performance and breeding in horses, and it is also important in terms of oral and skin integrity in the pet sector with possibly causing other secondary diseases in of cats and dogs. There are many studies in the world and in our country that have examined these aspects. In these studies, papillomaviruses are investigated in different animal species and various factors of the virus (insulin growth factor I, II, and viral genes E2, E5, E6 etc.) are also questioned. (Alcigir et al. 2016; Oguzoglu et al. 2017; Alçığır and Timurkan 2018). According to the results of the study, BPV-12 was detected for the first time in our country; EcPV-2 was detected from a cattle penis lesion supporting the evidence cross species infection not only occurs from cattle to horses, but reverse route can also be possible for the first time; p53 immunopositive reactions are most prominent in *Deltapapillomaviruses* namely BPV-1 and BPV-2. p53 findings of the study conclude these genotypes have higher risk of causing malign transformation and supports these genotypes are high risk BPVs. Further studies are needed, including experimental studies, especially on explaining cross species infection transmission mechanisms.

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Declarations

Conflict of interest The authors declare no conflicts of interest.

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