PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 43:e07150, 2023
DOI: 10.1590/1678-5150-PVB-7150

Original Article
Livestock Diseases

Searching bovine papillomavirus presence in lesions seen on teats of cows[1]

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ABSTRACT.- Özmen G. & Kale M. 2023. **Searching bovine papillomavirus presence in lesions seen on teats of cows**. *Pesquisa Veterinária Brasileira 43:e07150, 2023*. Department of Virology, Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy Üniversitesi, Burdur, Turkey. E-mail: drmkalex@yahoo.com

The samples were taken from 106 cows with various-looking lesions on their teats and ranged in age from 2 to 8 years. Enzyme-linked immunosorbent assay (ELISA) antigen (Ag) positive for the bovine papillomavirus (BPV) was found in 59 (55.7%) blood serum samples. PCR using FAP59/64 primers was positive for 24 (22.6%) samples. BPV-2 (40, 37.7%), BPV-6 (28, 26.4%), BPV-8 (30, 28.3%), BPV-9 (36, 34%), BPV-10 (32, 30.3%), and BPV-12 (22, 20.8%) were found in a PCR type-specific analysis of single and mixed type teat warts. The highest positivity was observed in BPV-2, BPV-9 and BPV-10 in flat and round forms, BPV-6, BPV-10, BPV-12, and mixed types in rice grain-cauliflower forms, BPV-9 and mixed types in filiform in the distribution of types based on the macroscopic appearance of teat lesions. As for the distribution of BPV types according to age, the most BPV-2 types were found in the age group of two years, the most BPV-10 types in the age group of three years, the most BPV-9 types in the age group of four years, the most BPV-8+BPV-12 types in the age group of five years, and the most mixed types between the ages of six and eight years. The existence of the virus was then checked using electron microscopy on the chosen samples (at least one investigation was conducted), and it was positively identified using BPV type-specific primers. The authors concluded that BPV detection using an ELISA (Ag) test from blood serum samples was shown to be less sensitive than BPV type-specific PCR from wart samples.

INDEX TERMS: Bovine papillomavirus, pathology, diagnosis, teat, virology, cows.

INTRODUCTION

Papillomatosis, often known as skin warts, is a viral illness that affects both domestic and wild animals and people. The skin condition known as papillomatosis is characterized by benign proliferative tumors with complex pathophysiology and etiology and epithelial proliferation. Although host and environmental factors can influence the development of warts in various body regions, papillomaviruses (PVs) that are mostly host and tissue-specific are typically to blame for the disease. Papillomas are typically found on the skin, teats, and mucous surfaces of cattle (Villiers et al. 2004, Jelinek & Tachezy 2005, Campo 2006). Papillomavirus (PV) lesions on the teats and nipples of dairy calves cause mastitis, low milk yield, blind and unusable teats, teats impossible to fit into milking machines due to deformities, little milking due to pain, and therefore significant economic losses. Additionally, because papillomas are damaged, they serve as a foundation for secondary infections, which significantly raises the prevalence of the illnesses and their cost of care (Kale 2020).Clinical features and histopathological applications are mostly used to diagnose PV infection (Turk et al. 2005, Betiol et al. 2012). The most popular technique for identifying viral DNA in tissue and blood samples from BPV-infected animals is the polymerase chain reaction (PCR) assay (Tomita et al. 2008, Wobeser et al. 2012, Ataseven et al. 2016). It can be used in bovine papillomavirus (BPV) type detection, genotype-specific or degenerate primer use, in-situ hybridization sequencing and phylogenetic analysis, immune fluorescence, Western blot, Southern blot, Dotblot, reverse blot, and PCR restriction fragment length polymorphism diagnosis (Notomi et al. 2001, Kidney & Berrocal 2008, Leto et al. 2011, Roperto et al. 2012, Carvalho et al. 2013, Grindatto et al. 2015). Additionally, successful diagnostic techniques include immunohistochemical analysis and transmission electron microscopy (TEM) (Turk et al. 2005, Postey et al. 2007, Catroxo et al. 2013, Araldi et al. 2014, Gallina et al. 2020).

[1] Received on September 4, 2022.
Accepted for publication on October 3, 2022.
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In this study, we used enzyme-linked immunosorbent assay (ELISA), PCR, histopathology, immunohistochemistry, and electron microscopy methods to identify the presence of BPV in lesions macroscopically different on cow teats.

MATERIALS AND METHODS

Animals and management. Tables 1 and 2 show blood and tissue samples from 106 female cows owned by the general public in Burdur center and its surrounding regions that ranged in age from 3.81 to 1.38 (2 to 8 years) and had various lesions on their teats (flat and round, rice grain-cauliflower, filiform).

ELISA (Ag). The MyBioSource company's qualitative sandwich ELISA (Ag) kit (Qualitative BPV ELISA, Catalog # MBS109004, San Diego, USA) was used to find the presence of BPV in blood serum samples. The test was performed according to the kit's instructions.

PCR. Using the DNeasy Blood &Tissue Kit, virus DNA was isolated from nipple lesions (Qiagen, Germany). FAP59/64 and MY11/09 consensus primers were utilized to detect various BPV types (Silva et al. 2016). Except for BPV types 1 through 14, type 7, and Table 3, Silva et al. (2016) recommended BPV type-specific primers were utilized. According to Silva et al. (2016), both primers (consensus and kinds) were administered by mixing them. Two percent (2%) Tris-acetate buffer (TAE), agarose gel electrophoresis, and ethidium bromide stain were used to demonstrate the amplification products (Silva et al. 2016).

Histopathology. The papillomatous teat lesions were fixed in 10% neutral buffered formalin, routinely processed for histopathology and stained with hematoxylin and eosin (HE).

Immunohistochemistry. The streptavidin-biotin peroxidase complex method was used to stain the sections placed onto polylysine slides for the immunoperoxidase procedure. Mouse and Rabbit Specific HRP/DAB IHC detection kit-micro-polymer (ab236466) from Abcam, Cambridge, England, was used as a secondary kit and for the BPV antiserum (Anti-HPV antibody (BPV-1/1H8+CAMVIR) (ab2417), 1/50 dilution). The tissues were then covered with primer antibodies without being washed, allowed to sit for two hours at room temperature, washed twice with PBS, and then subjected to a 10-minute treatment with the mouse complement. After this step, they received two 10-minute PBS washes. The tissues underwent a 15-minute incubation with Goat Anti Rabbit HRP conjugate followed by four PBS washes. After washing, the DAB substrate (3,3 diaminobenzidine tetrahydrochloride) was applied for 10 minutes, and the procedure was then completed by anti-staining with Harris hematoxylin. The lamella was transparentized in xylol, closed with an adhesive (EntellanTM-Merck), and examined under a light microscope after the tissues through the alcohol series had been dehydrated (Olympus CX41). Using the Database Manual Cell Sens Life Science Imaging Software System, microphotography and morphometric analysis were conducted (Olympus Corporation, Tokyo, Japan).

Transmission electron microscopy (TEM). This approach was used to conduct an electron microscopic investigation on 12 samples, one of which had teat lesions caused by BPV types 1 through 13. The samples were obtained and subjected to primer fixation at 4°C for 24 hours in 2.5% glutaraldehyde containing 0.1M phosphate buffer. Three phosphate buffer washes lasting 15 minutes each were then performed on the samples. Afterward, the tissues were rinsed three times with phosphate buffer before receiving secondary fixation for 2 hours in a rotator at room temperature in 1% osmium tetroxide. The samples were taken under transparentization for 30 minutes (twice) in propylene oxide to remove the excess water (dehydration) from the tissues. This was accomplished by passing the samples through ethyl alcohol series twice in specific periods at increasing temperatures and 4°C (30%, 50%, 70%, 90%, 96%, 100%, final wash, and subsequent applications at room temperature). The tissues were immersed in pure Araldite for one night, retained in

Table 1. Distribution of papilloma samples according to macroscopic appearance

Macroscopic appearance	Numbers (%)	Mean	Standard deviation	Mean 95% CI	
				Low	High
Flat and round	50 (47.17)	3.36	1.481	2.94	3.78
Ricegrain-cauliflower	10 (9.43)	4.00	0.943	3.33	4.67
Filiform	46 (43.40)	4.26	1.201	3.90	4.62
TOTAL	106 (100)	3.81	1.381	3.55	4.08

Table 2. Distribution of samples according to age

Age (year)	Numbers	Percentage (%)
2	18	16.98
3	32	30.19
4	26	24.53
5	18	16.98
6	8	7.54
7	2	1.89
8	2	1.89
Total	106	100

Table 3. Bovine papillomavirus (BPV) type specific primers

Primers	Sequences	Fragment size (bp)	Amplified region
BPV-1	F-5' GGA GCG CCT GCT AAC TAT AGG 3' R-5' ATC TGT TGT TTG GGT GGT GAC 3'	301	L1 gene
BPV-2	F-5' GTT ATA CCA CCC AAA GAA GAC CCT 3' R-5' CTG GTT GCA ACA GCT CTC TTT CTC 3'	164	L1 gene
BPV-3	F-5' CAG TCA ATT GCA ACT AGA TGC C 3' R-5' GGC TGC TAC TTT CAA AAG TGA 3'	216	L1 gene
BPV-4	F-5' GCT GAC CTT CCA GTC TTA AT 3' R-5' CAG TTT CAA TCT CCT CTT CA 3'	170	E7 gene
BPV-5	F-5' GGC ATG TAG AGG AAT ATA AGC 3' R-5' TTC TCT GAG ATC AAT ATT CC 3'	262	L1 gene
BPV-6	F-5' TTA GAG ACC TGG AAC TTG GG 3' R-5' TAC GCT TTG GCG CTT TTT TGC 3'	294	L1 gene
BPV-8	F-5' TAG AGG ACA CAT ACC GCT TCC AAA GC 3' R-5' TTT GCG AGC ACT GCA GGT GAT CCC 3'	196	L1 gene
BPV-9	F-5' AAA GAG CAA ATC GGG AGC ACC 3' R-5' AAC TAA TGA CCC ACT AGG GCT CC 3'	264	L1 gene
BPV-10	F-5'AAG GCA TTT GTG GTC TCG AGG 3' R-5' CTA AAG AAC CAC TTG GAG TGC C3'	148	L1 gene
BPV-11	F-5' TGC AGA CAC TCA ACC AGG AG 3' R-5' CCA TAA GGG TCG TTG CTC AT 3'	197	L1 gene
BPV-12	F-5' AAA GCT GAA CCA TGC AAA CC 3' R-5' TAA CAA TGT CAA GGG GCA CA 3'	159	L1 gene
BPV-13	F-5' CCA ACC CCA GTA AGC AAG GT 3' R-5' AAG AGG TTG ACC TCG GGA GA 3'	288	L1 gene
BPV-13	F-5' CAC TGC CAT TTG GTG TTC TT 3' R-5'AGC AGT CAA AAT GAT CCC AA 3'	153	E5 gen
BPV-14	F-5'GGA ACA AAC CTC ACA ATC AC 3' R-5'CCA GTT CTC TAA TAC TGA GG 3'	195	L1 gene

a rotator for two hours with a propylene oxide-Araldite mixture at a rate of one to one, embedded in Araldite the following day, and polymerized for 48 hours at 60°C.

The resulting blocks were sliced using an ultramicrotome (Leica Ultracut R) with a thickness of 700nm, dyed with toluidine blue, and the light microscope was used to investigate the surface areas that might contain biofilm (Olympus BX50). The tissues were retrimmed, and their precise thin sections were made in an ultramicrotome with a thickness of 60nm after the locations required to be monitored in TEM were identified. These sections were put into copper grids with a mesh size of 300, stained with uranyl acetate lead citrate, and then analyzed in a TEM made by JEOL, model JEM 1220. These procedures were carried out in the Electron Microscopy Monitoring and Analysis Unit at Akdeniz University's School of Medicine (TEMGA).

Statistical analysis. It was analyzed how papilloma BPV types were distributed based on macroscopic appearance and how they were distributed based on age (%). The one-way ANOVA test was used to investigate the relationship between age and the macroscopic types of papillomas. Using an independent sample test, the relationship between BPV type and age that causes papillomas in cattle was examined. SPSS Statistics 17.0 was used to analyze the data (IBM Software). Paired t-test was carried out using the correlation (Pearson) analysis Minitab 19 program to compare the test findings of BPV ELISA (Ag), PCR, histology, and immunohistochemistry assays.

RESULTS

ELISA (Ag)

Fifty-nine (59, 55.7%) blood serum samples used in the test were found to be BPV (Ag) positive.

PCR

Consensus primers FAP59/64 and MY11/09 were utilized in PCR for a broad range of BPV-type detection. Of the 106 samples, FAP59/64 positive was found in 24 (22.6%). No MY11/09 consensus primer was found in any of the samples. BPV-1 (L1), BPV-2 (L1), BPV-3 (L1), BPV-4 (E7), BPV-5 (L1), BPV-6 (L1), BPV-8 (L1), BPV-9 (L1), BPV-10 (L1), BPV-11 (L1), BPV-12 (L1), BPV-13 (L1), BPV-14 (L1), and BPV-14 (L1) were used. As a result, whereas BPV-14 positivity could not be discovered in any of the samples, BPV positivity was generally found in all 106 samples. In the samples, there were 12 (11.3%) BPV-9, eight (7.5%) BPV-10 and eight (7.5%) BPV-2 with the highest single-type positivity distribution. The distribution of double-positive with the highest frequency was found to contain four (3.7%) BPV-6+BPV-9, four (3.7%) BPV-8+BPV-10, and four (3.7%) BPV-8+BPV-12. BPV-2+BPV-6+BPV-9 was the triple-type positivity distribution that was found to be the highest. Positive test results for types 4, 5, and 6 were found in the other samples (Table 4). Regarding teat warts, the following were found to be more prevalent in general distribution: BPV-2 (40, 37,7%), BPV-6 (28, 26,4%), BPV-8 (30, 28,3%), BPV-9 (36, 34.0%), BPV-10 (32, 30,3%), and BPV-12 (22, 20,8%) (Table 5).

According to their macroscopic appearance, teat lesions were divided into filiform, rice-grain form, and flat and round shapes. The most common forms of the BPV-2 (12%), BPV-9 (8%) and BPV-10 (12%) types were flat and round, followed by rice grain forms for the BPV-6 (20%), BPV-10 (20%), BPV-12 (20%), BPV-8+BPV-9 (20%), and BPV-3+BPV-6+BPV-8+BPV-11 (20%) types. The most common forms of BPV-9 (17.4%), BPV-6+BPV-9 (8,7%) and BPV-8+BPV-12 (8,7%) in filiform.

Table 4. Distribution of PCR and FAP59/64 positives in single and mix types

Single and mix types	Positives n(%)
BPV-1	2 (1.9)
BPV-1, 12	2 (1.9)
BPV-1, 2, 5, 12	2 (1.9)
BPV-2 and FAP59/64	8 (7.5)
BPV-2, 6 and FAP59/64	2 (1.9)
BPV-2, 8 and FAP59/64	2 (1.9)
BPV-2, 9	2 (1.9)
BPV-2, 10 and FAP59/64	2 (1.9)
BPV-2, 12 and FAP59/64	2 (1.9)
BPV-2, 4, 6 and FAP59/64	2 (1.9)
BPV-2, 6, 12	2 (1.9)
BPV-2, 6, 9	4 (3.7)
BPV-2, 8, 9	2 (1.9)
BPV-2, 8, 10, 12	2 (1.9)
BPV-2, 3, 9, 10	2 (1.9)
BPV-2, 8, 9, 10	2 (1.9)
BPV-2, 3, 6, 10, 12	2 (1.9)
BPV-2, 5, 8, 9, 10, 13	2 (1.9)
BPV-3, 11 and FAP59/64	2 (1.9)
BPV-3, 6, 8, 11	2 (1.9)
BPV-3, 8, 9, 10	2 (1.9)
BPV-6	4 (3.7)
BPV-6, 8 and FAP59/64	2 (1.9)
BPV-6, 9	4 (3.7)
BPV-6, 10	2 (1.9)
BPV-6, 10, 12 and FAP59/64	2 (1.9)
BPV-8	2 (1.9)
BPV-8, 9	2 (1.9)
BPV-8, 10	4 (3.7)
BPV-8, 11	2 (1.9)
BPV-8, 12	4 (3.7)
BPV-9	12 (11.3)
BPV-9, 10	2 (1.9)
BPV-10	8 (7.5)
BPV-10, 11, 12	2 (1.9)
BPV-11	2 (1.9)
BPV-12	2 (1.9)
TOTAL	106 (100)

Table 5. General distribution of single or mix types at all

BPV types*	Positives	Percentage (%)
BPV-1	6	5.7
BPV-2	40	37.7
BPV-3	9	8.5
BPV-4	2	1.9
BPV-5	4	3.8
BPV-6	28	26.4
BPV-8	30	28.3
BPV-9	36	34.0
BPV-10	32	30.3
BPV-11	10	9.4
BPV-12	22	20.8
BPV-13	2	1.9

* Total of single or mix types.

The study assessed the distribution of BPV types by age. The prevalence of BPV-2 type was highest in the age groups of two (33.3%), three (12.5%), four (23.1%), five (22.3%), and eight (BPV-8+BPV-12) and twelve (BPV-9+BPV-10), as well as in the age groups of four (BPV-2+BPV-4+BPV-6), eight (BPV-2+BPV-8+BPV-9+BPV-10), seven (100%) and eight (100%). It was found that there is a greater chance that BPV types will combine over time. Using a one-way ANOVA (F – 5.66 and p=0.005) test, it was possible to determine the relationship between the macroscopic forms of papillomas in cattle and age. The age at which rice grain form papillomas were observed and the age at which other form papillomas were observed did not statistically differ (p>0.05).

Histopathology

In the 106 tissue samples that were evaluated, 98 papillomas (92.45%) and eight fibropapillomas (7.55%) were identified. Teat tissues with fibropapillomas included BPV-6, BPV-8, BPV-9, and BPV-12. The epidermis thickened dramatically, and the keratin layer increased in papilloma instances. In the keratin layer, nucleated acanthotic cells were seen. Parakeratosis and acanthosis were seen. In epidermal cells, spongiosis and balloony degenerations were commonly observed. In many instances, basophilic inclusion particles in these cells attracted attention. Epidermis degradation and ulcers were frequently seen, especially in huge bodies. In these regions, there were infiltrations of inflammatory cells with polymorphonuclear cells primarily made up of neutrophil leucocytes.

Additionally, bodies with erosion and ulcers usually exhibited dense bacterial clusters. The quantity and size of keratohyalin granules in keratinocytes increased in papilloma-affected areas. Coilocytes with extensive cytoplasmic vacuoles and an eccentric, hyperchromatic nucleus were frequently observed in cells of the stratum spinosum and granulosum. In numerous instances, the basal layer displayed significant coloration. Extreme proliferations in the dermis were seen, as well as rete ridges where the dermis had formed indentations and protrusions toward the epidermis. Most of the ligament comprises bundles of collagen and fibroblast filaments stretched in various directions. Attention was also drawn to the rise in mitotic activity in these regions. Extreme veining and bleeding were seen in certain bodies. Proliferations were occasionally detected in the dermis that resembled those in the epidermis, and these cases were classified as fibropapillomas (Fig.1-4).

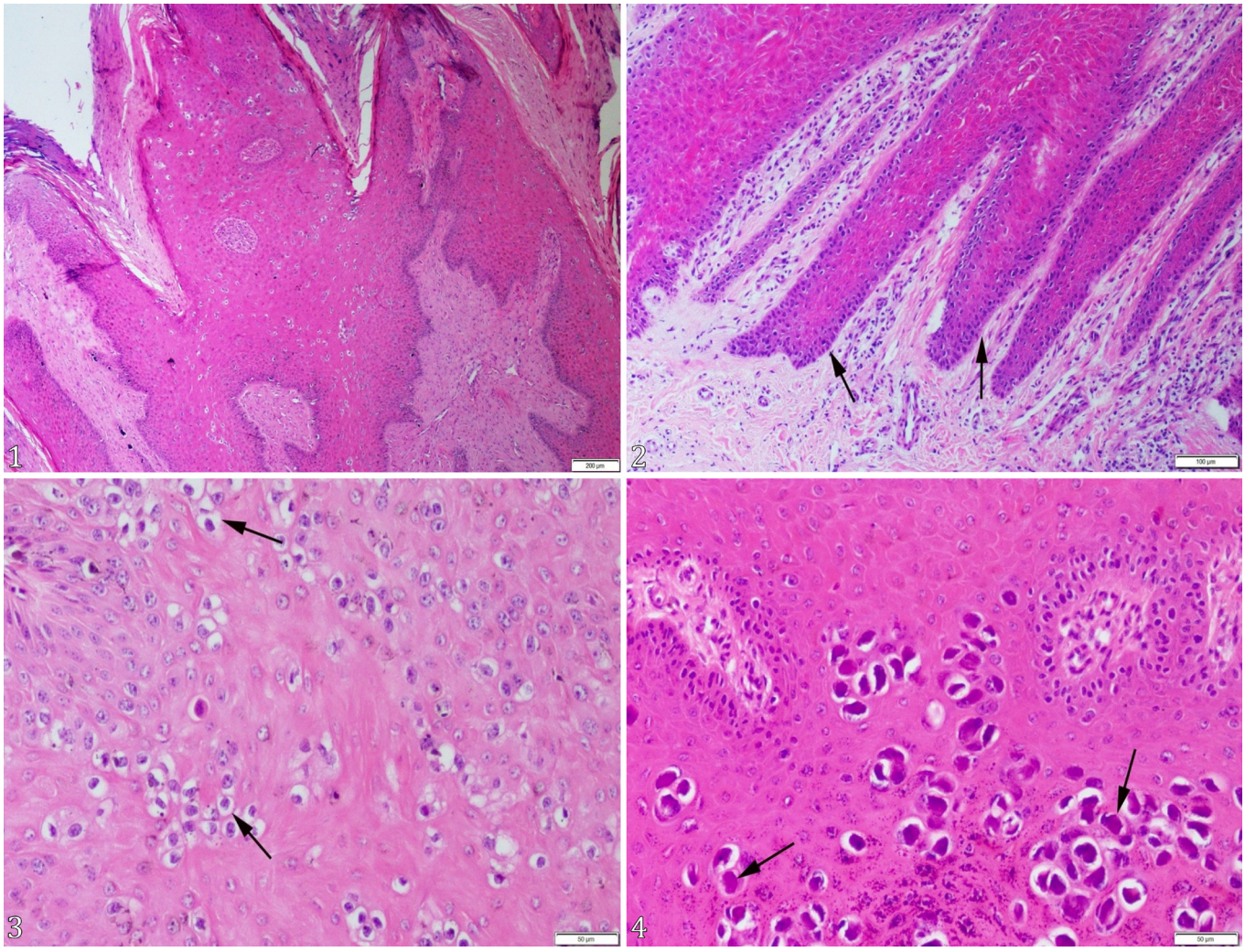

Fig.1-4. (**1**) Image of a typical papilloma, thickening in epidermis and keratin layer. HE, bar = 200µm. (**2**) Histopathological appearance of papilloma and rete ridges (arrows). HE, bar = 100µm. (**3**) Large numbers of koilocytes with vacuole (arrows). HE, bar = 50µm. (**4**) Inclusion particles in koilocytes (arrows). HE, bar = 50µm.

Immunohistochemistry

Positive immunolabeling was observed in the epidermis, keratin layer, and dermis (Fig.5).

TEM tissue follow-up

During EM examinations applied on teat papilloma lesion samples selected by positive detection with BPV type-specific primers (one examination was performed out of a single sample for BPV-1, 2, 6, 8, 9, 10, 11 and 12; a single one out of mixed samples for BPV-3, 4, 5 and 13), presence of virus was observed. During EM examinations, BPV particles were detected to have been located within intracytoplasmic and/or intranuclear areas in epithelium cells (Fig.6-7).

Comparing ELISA (Ag), PCR, histopathology and immunohistochemistry test results

Histopathological analyses and immunohistochemical assays on teat lesions in the study revealed positivity in all the samples discovered with BPV-type specific primers. In the study, the results from tissue samples investigated by PCR-histopathology-immunohistochemistry tests and blood serum samples analyzed by ELISA (Ag) tests were compared as an assessed and a reference test. As a result, when they were compared using the BPV ELISA Ag (evaluated test) and the PCR-histopathology-immunohistochemistry assays (reference test), sensitivity was found to be 55.7%, specificity was 100%, and correlation was 23.3% (p=0.767, insignificant). Because of this, when they were compared as BPV ELISA Ag (reference test), and PCR-histopathology-immunohistochemistry assays assessed test), sensitivity was found to be 100%, specificity was found to be 50%, and correlation was found to be 23.3% (p=0.767, insignificant).

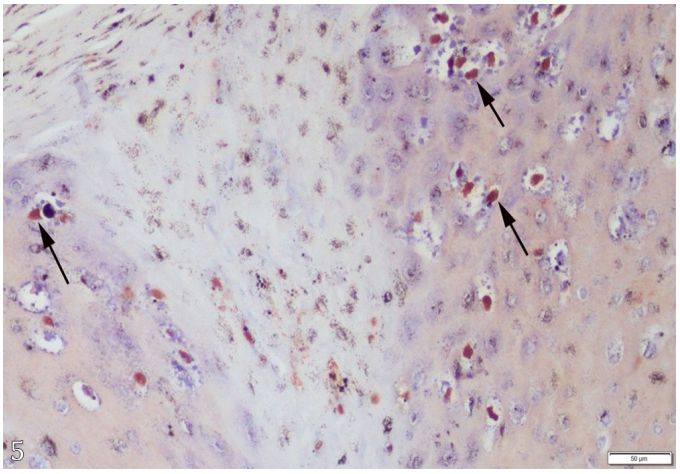

Fig.5. Bovine papillomavirus (BPV) positive immune reaction (arrows), streptavidin-biotin peroxidase method. HE, bar = 50µm.

DISCUSSION

Cows' teat lesions may develop due to infectious and non-infectious reasons (Hillerton et al. 2001). One of the major illnesses impacting bovine health is teat papillomatosis, and lesions, particularly those that occur on the teats, may result in large financial losses in the milk sector (Campo 2003).

The BPV-ELISA test was developed by El Shazly et al. (1985). It was established that the findings of this test were comparable to those of negative marked EM tests and paraffined peroxidase-anti-peroxidase staining of the original fibropapillomas in terms of virus detection. Additionally, this test has been shown to be quick and can detect viral loads as low as 1ng/ml. A blood sample from a cow with a wart body on the maxillary area was investigated by Kale et al. (2018) for the purpose of diagnosing BPV using a commercial, qualitative BPV ELISA test kit. In their research, they found a favorable result. A dairy cow with severe teat warts was treated by Kale et al. (2019) using podophyllin and an autologous vaccination. They used ELISA to check for the presence of BPV (Ag) in the animal's blood serum and found a positive result. Each blood serum sample collected from the animals with teat papillomas in our investigation was subjected to a BPV (Ag) ELISA test. This test kit was prepared as a sandwich ELISA to identify the presence or absence of BPV in serum, plasma, and other biological fluids. Fifty-nine (59) blood serum samples (55.7%)

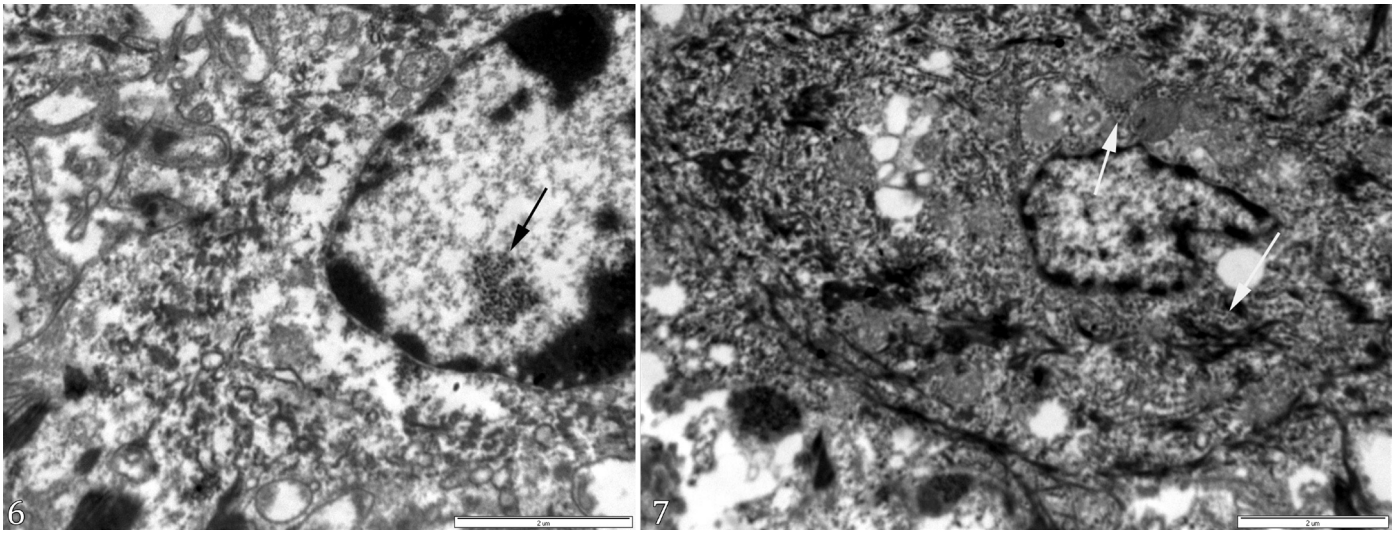

Fig.6-7. (**6**) Electron microscopy (EM) appearance of intranuclear virus particles (arrow) in teat epithelium cells. Bar = 2µm. (**7**) EM appearance of intra-cytoplasmic virus particles (arrows) in teat epithelium cells. Bar = 2µm.

were determined to be BPV (Ag) positive after the test was run. Aside from a few case studies, no virological study on field BPV presence ELISA blood serum samples were found during literature searches.

PCR and consensus primers designed for the genes encoding the L1, L2, E6, and E7 proteins allowed for the detection and characterization of PVs (Manos et al. 1989, Forslund et al. 1999, Ogawa et al. 2004, 2007, Lindsey et al. 2009). Primer sets FAP59/64 and MY09/11 have been used to detect PV in people, cattle, and other animals (Manos et al. 1989, Forslund et al. 1999, Antonsson & Hansson 2002, Ogawa et al. 2004, 2007, Lindsey et al. 2009). These primers were designed based on the HPV L1 gene's protected regions (Rai et al. 2011). FAP59/64 primers might be used for DNA detection. However, PCR analysis will be performed using MY09/11 primers unless samples are amplified. L1 gene, overall molecular identification, and phylogenetic analysis of BPVs could all be done using both primer-amplified sequence sets (Forslund et al. 1999, Ogawa et al. 2004, Silva et al. 2016). Dağalp et al. (2017) detected BPV positivity with both FAP59/64 and MY09/11 primer sets in 22 of 35 samples (62.9%); seven (20%) samples with FAP59/64, five samples (14.3%) with MY09/11 and 34 (97.1%) samples with both degenerated and type-specific primers. Zhu et al. (2019) detected positivity with FAP59/64 primers in all the tissue (48) and swap (36) samples taken in Holstein managements with teat warts while they observed PCR negativity with MY09/11 primers. They mostly detected BPV-10 with BPV-type specific primers in these samples. This study found FAP59/64 positivity in 24 (22.6%) of 106 samples. MY11/09 consensus primer could not be detected in any samples. In the study, FAP59/64 primers were detected together with specific primers (BPV-2, BPV-3, BPV-4, BPV-6, BPV-8, BPV-10, BPV-11 and BPV-12). Although Grindatto et al. (2015) claimed that type-specific or degraded (FAP59/64, for example) primers were utilized in BPV identification, quite a few discrepancies were determined when these two systems were compared. According to Dağalp et al. (2017), BPV type-specific primers are more sensitive than degenerated ones, and degenerated primers are ineffective in samples containing a variety of BPV mixed types. Silva et al. (2013) suggested that although FAP59/64 degenerated primers were ideal for basic viral types and new PV, they had a lower sensitivity than other specific primers and were unable to detect some viral types (such as BPV-4 and BPV-9) and that this was because they were created with the HPV target in mind. The effectiveness of consensus primers may be influenced by the quantity, location, and stability of the mismatch, according to Qu et al. (1997). The same study confirmed that there were variations in type-specific amplification efficiency that were caused by a degeneracy synthesis in the consensus primers. In situations that regularly occur in the field, degraded primers could not be found in comparable samples but with many types. On the other hand, it was not economical to convey scanning analysis using type-specific in areas where epidemiological scenarios were not realized, and viral types could not be detected.

According to primers specific to the BPV type used in this investigation, BPV positive was generally observed in all 106 samples. However, BPV-14 positivity could not be detected in any of the samples. Teat papillomas were classified into single categories in general distribution, and BPV-2 (8, 7.5%), BPV-9 (12, 11.3%), and BPV-10 (8, 7.5%) were found to be more prevalent. The presence of BPV-13 was found in cutaneous papillomas (Lunardi et al. 2013, Silva et al. 2015). The two most prevalent kinds of BPV in epidemiological studies on the disease were BPV-1 and BPV-2 (Melo et al. 2014, Cota et al. 2015). Jana (2015) considered BPV-1 and BPV-2 potential causes of cutaneous and teat papillomas. After conducting applied investigations, it was found that BPV-6 and BPV-9 were frequently responsible for teat papillomatosis (Hatama et al. 2009). Only BPV-10 presence was found in cows in Indian dairy management; other types were not found. Dağalp et al. (2017) frequently found BPV-6, BPV-7, BPV-9 and BPV-10 in teat papillomas. In our study, BPV-2, BPV-9 and BPV-10, from which single types were obtained in teat papillomas, correspond with the studies mentioned above.

In our study, the general distribution, where mixed types (double and triple) were observed together, had higher prevalences of BPV-2, BPV-6, BPV-8, BPV-9, BPV-10, and BPV-12. Teat papillomas contained BPV-6, BPV-7, BPV-8, BPV-9, and BPV-10, according to Lunardi et al. (2016). In mixed infections, they determined BPV-7+BPV-10, BPV-6+BPV-9, BPV-6+BPV-10, and BPV-8+BPV-10. In 44 teat papillomas, BPV-7, BPV-9, and BPV-10 DNA positive were found by Savini et al. (2016). Using conventional PCR, Jana (2015) found BPV-1, BPV-2, BPV-5, BPV-9, and BPV-10 in teat cutaneous papillomas. Bianchi et al. (2020) found BPV-4, BPV-6, BPV-7, BPV-8, BPV-9, BPV-10, BPV-11, BPV-12 types in 27 samples in 73 teat papilloma lesions they collected from a slaughterhouse, in 6 putative BPV types previously stated in 17 samples and 10 new BPV types in 15 samples.

According to the distribution of types in teat lesions based on their macroscopic appearance, BPV-2, BPV-9 and BPV-10 were primarily observed in flat and round forms, BPV-6, BPV-10, BPV-12, BPV-8+BPV-9, BPV-3+BPV-6+BPV-8+BPV-11 in rice-grain forms, and BPV-9, BPV-6+BPV-9, BPV-8+BPV+12 in filiform. In this study, the rice-grain form was able to exhibit a variety of microscopic appearance characteristics in lesions. As a result, many BPV-co-infection types were detected in tissues. In rice-grain forms, the rate of the greatest single-type virus (60%) was found to be greater. The BPV-2, BPV-10, and BPV-9 in filiform were shown to have the maximum positive. However, filiform (69.6%) and flat and round shapes (60%) had the highest percentages of mixed-type viruses. Based on the origin and pathology of cutaneous papillomas, thick, congested skin develops. Depending on where they are, they present varied exteriors. They turn hard, dry, and horny when coated in the epidermis. Most papillomas feature epidermal proliferations, which have keratotic surfaces that resemble rice grains (Jana 2015).

Additionally, it was stated that BPV infections could result in co-infections with other types and could manifest themselves in different combinations in lesions of a similar nature (Claus et al. 2008, Schmitt et al. 2010, Carvalho et al. 2012, Batista et al. 2013, Bocaneti et al. 2016). According to Lunardi et al. (2016), it was challenging to establish a relationship between BPV types and anatomical tropisms of skin papillomas based on empirical observations, and virus types could not be restricted to a particular anatomical location in cattle. Even though they could not identify more than one type in a single lesion, Bertagnolli et al. (2020) could not establish a link between BPV types and the macroscopic appearance

or anatomical locations of lesions. They claimed that since co-infections have become implicated, as other researchers had asserted, this case took place (Carvalho et al. 2012, Batista et al. 2013, Daudt et al. 2016). They claimed that the infections could not have been caused by the viruses found and that persistent infections might arise in mixed infections due to immune suppression (Batista et al. 2013, Carvalho et al. 2013). Despite the above-mentioned reasons, numerous researchers (McMurray et al. 2001, Villiers et al. 2004, Hatama et al. 2008) also claimed that papillomas varied depending on the specificity of tissues and that this was influenced by the viral genotype. This study uses findings that were first locally noticed in one place. We urge those involved to do research across a wider animal population.

According to age groups, the BPV-2 type was most prevalent in the two to four-year-old age range, followed by the BPV-10, BPV-9, BPV-8+BPV-12, and mixed kinds in the age ranges of six to eight. Compared to filiform warts, flat and round warts were more common earlier in life (p=0.003). There was no significant statistical difference between the age at which warts in the rice-grain form were observed and the age at which the other types of warts were observed (p>0,05). Numerous experts stated that animals between six months and 10 years might develop papillomas (Jelinek & Tachezy 2005, Turk et al. 2005, Wosiacki et al. 2005, Borzacchiello et al. 2009). Similarly, Smith (1996) claimed that all age groups of cattle were affected by the illness, even though incidences of papilloma were only observed in cattle younger than two years of age. According to Meischke (1979), there were no statistically significant differences in BPV prevalence or diversity between older and younger calves with teat papillomas. According to Jana (2015) research, animals can develop cutaneous papillomas between the ages of six months and four years, while teat papillomas can develop up to the age of eight. In certain investigations, papillomas were noted in animals between the ages of six and 60 months (Çimtay et al. 2003, Atasever et al. 2005). Additionally, the incidence of cutan warts was highest in young animals (Kumar 2012), and adult cattle had the highest incidence of BP, followed by heifers and calves (Jubb & Kennedy 1970). However, young ones were more sensitive to this infection than the elders. The freshly formed immune system in the young, maternal antibody loss due to weaning, sensitivity to parasite infections, the effectiveness of stress factors, ectoparasites (tick infestations or acariasis), and alkaline skin pH could all be contributing factors. In our investigation, we conducted BPV detection in all age groups, and the likelihood of mixed BPV-type appearance increased as time went on. Higher teat papilloma formation was observed by Jana (2015) and Sharma et al. (2005) in cows and buffalos with high calving intervals (pluriparious) throughout their advanced lactation periods in the winter and autumn seasons. Stress factors like pregnancy, advanced age, and lactation were thought to be important in developing infections (Jana 2015).

Ninety-eight (92.45%) papillomas and eight (7.55%) fibropapillomas were detected in the examined 106 tissue samples. In the teat tissues undergoing fibropapilloma detection, BPV-6, BPV-8, BPV-9 and BPV-12 were found. The epidermis became extremely thick in papilloma cases, and the keratin layer increased. Acanthosis and parakeratosis were apparent in the keratin layer. Spongiosis and balloony degenerations frequently appeared in squamous cells in the epidermis. Coilocytes were frequently seen in cells in the stratum spinosum and granulosum. Severe proliferations and rete ridges formed as indentation and bulge forms by dermis were observed. The ligament was mostly formed by fibroblast and collagen fibers stretched towards various directions in bundles. In some cases, proliferations were seen in the epidermis and dermis, and such cases were considered fibropapilloma. In general, within the histopathological findings related to PV, acanthosis, hyperplasia of the spinal epithelial layer, koilocytosis, hypergranulosis, hyperkeratosis, parakeratosis, papillomatosis, transformed fibroblasts, and even vacuoles degeneration of skin spinosum layer might be observed (Turk et al. 2005, Anjos et al. 2010, Marins & Ferreira 2011, Timurkan & Alcigir 2017). Among the prominent findings were hyperkeratosis and acanthosis in PV teat lesions in cows and the proliferation of fibroblasts in fibropapillomas (Scagliarini et al. 2016). In this study, our histopathological study findings in teat lesions were similar to those of the researchers mentioned above (Maeda et al. 2007, Hatama et al. 2009, Hatama 2012, Beytut 2017).

In this study, positive immunolabeling was observed in the epidermis in sections (106) stained with BPV serum. Also, it was detected in the keratin layer. In some cases, a positive reaction was also found in capillary veins in the dermis. Findings and results in our study found by immunohistochemical diagnosis in teat papillomas (Jelinek & Tachezy 2005, Maeda et al. 2007, Hatama et al. 2009, Catroxo et al. 2013, Babu et al. 2020, Beytut 2017) were found similar to those by other researchers.

The presence of the virus was detected during the EM exams performed on teat papilloma lesions that had been identified as positive by BPV-type specific primers (one examination was performed as single for BPV-1, 2, 6, 8, 9, 10, 11 and 12, and mixed for BPV-3, 4, 5 and 13). In this study, EM analyses revealed the presence of BPV particles in intracytoplasmic and/or intranuclear regions of epithelial cells. In their investigation employing EM for teat lesions, Maeda et al. (2007) found viral particles within the nucleus of stratum granulosum cells in the epidermis, where BPV-6 and other unclassified BPV types were identified. Melo et al. (2015) determined BPV virus particles packed in the cytoplasm of skin lesion samples from cattle, which was not the case in this instance.

CONCLUSIONS

In this investigation, it was discovered that teat papillomas in dairy cattle were caused by BPV infection. Direct application of BPV-type specific primers was shown to have produced superior outcomes for diagnosis in papilloma tissues. The most common BPV types in teat papillomas included BPV-2, BPV-6, BPV-8, BPV-9, BPV-10, and BPV-12. BPV-2, BPV-9 and BPV-10 in papillomas with flat and round forms, BPV-6, BPV-10, BPV-12, and mixed types in papillomas with rice-grain forms, BPV-9 and mixed types in filiform papillomas all showed the highest positive. While the prevalence of single-type viruses was higher in rice-grain form papillomas, the prevalence of mixed-type viruses was higher in filiform papillomas.

BPV mixed types became common as time passed, and younger animals began exhibiting flat and round shapes. PCR, histology, immunohistochemistry, and EM applications revealed

parallelism in identifying teat lesions caused by BPV infection. The ability to identify BPV from blood serum samples using the ELISA (Ag) test was shown to be less sensitive.

Acknowledgments.- This study was summarized from a part of Ph.D. thesis submitted by Kale M. to the Burdur Mehmet Akif Ersoy University and supported by Burdur Mehmet Akif Ersoy University Scientific Research Projects Commission, project number: 0556-DR-18.

Conflict of interest statement.- The authors declare no conflict of interest.

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