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Ruminants

Investigation of the Presence of Papillomavirus in Bovine Testicles and Determination of γH2AX and Cytochrome C in Testicular Tissues Determined to be Infected

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ABSTRACT

This study aimed to detect the presence of bovine papillomavirus (BPV) in the testicular tissue of bulls over 1-year old by immunohistochemical, immunofluorescence and molecular assay targeting methods. In addition, γH2AX and cytochrome c expressions were evaluated by immunohistochemical and immunofluorescent methods in samples positive for BPV agent. In this study, 100 testicular specimens that did not show any macroscopic papilloma findings were collected. Testicular tissues were collected and examined using histologically and molecularly methods. In the present study, immunopositivity was detected in 6 (6%) samples with the IHC method and in 11 (11%) samples with the IF method. In addition, BPV positivity was detected in four (4%) samples by the PCR method and four (4%) samples determined to be BPV positive by PCR also showed immunopositivity in IHC and IF methods. γH2AX immunopositivity was seen in two of the BPV-positive samples by the IHC method, and γH2AX immunopositivity was observed in four of the samples determined to be positive for the BPV agent by the IF method. Cytochrome c immunopositivity was found in all the samples determined positive for the BPV agent. When the findings were evaluated, the presence of the BPV agent in the testicular tissues of cattle that did not have macroscopic papillomas was revealed for the first time. It was thought that the BPV agent caused double-stranded DNA breaks and increased cytochrome c expression.

1 | Introduction

Genetic and environmental factors are effective in bull fertility, a complex and economically important trait (Mishra et al. 2013). However, pathogenic agents are another important factor affecting fertility. The presence of these agents may cause infertility in bulls or spread through sperm transmission (Givens and Marley 2008). Several pathogenic agents can cause infertility in bulls or be transmitted through semen. Pathogen agents involved in infertility and transmission through semen can be categorised as viral, bacterial or protozoal agents. Some of these agents can directly affect fertilisation by causing diseases in the reproductive system or infecting spermatozoa (Givens and Marley 2008; Givens and Waldrop 2004; Wentink et al. 2000; Wrathall, Simmons, and Van Soom 2006).

Papillomaviruses are small, oncogenic, non-enveloped and round viruses belonging to the Papillomaviridae family (Van Regenmortel 2000). Bovine papillomavirus (BPV) causes bovine papillomatosis, which can be responsible for significant economic losses

due to growth retardation, weight loss and reduced milk production of affected animals (Campo 2003; Rector and Van Ranst 2013). The lesions occurring in the disease cause various losses in cattle breeding, depending on their width and anatomical location (Bertagnolli et al. 2020).

H2AX is a key factor in the repair process of damaged DNA. The amount of γH2AX in apoptotic cells is more than 10 times when compared to non-apoptotic cells (Tanaka et al. 2007). The number of phosphorylated H2AX molecules increases with the severity of the damage (Tanaka et al. 2007; Foster and Downs 2005). However, current studies show that the presence of γH2AX may be not only due to DNA breaks but also that free radicals may have an effect on this release (Jeong et al. 2017; Celeste et al. 2003).

Apoptosis is a non-inflammatory type of programmed cell death characterised by morphological changes such as cell shrinkage, nuclear condensation and plasma membrane blebbing (Kari et al. 2022). Cytochrome c is an electron transport chain protein located in the inner membrane of mitochondria. As a result of studies conducted in recent years, it has gained a very important place in the apoptosis process. The reason behind this is that the release of cytochrome c from the mitochondria to the cytoplasm is a marker of apoptosis; that is, the cell enters a period of irreversible loss (Kalpage et al. 2020).

Although previous studies within the scope of BPV have shown that the agent is located in the reproductive system and the agent is found in semen samples, there are no studies aimed at revealing the presence of the agent in testicular tissue. In our study, we aimed to reveal the presence of the BPV agent in testicular tissue samples, as well as to evaluate γH2AX and cytochrome c protein expressions in samples positive for the agent.

2 | Materials and Methods

2.1 | Study Material

A total of 100 testicular samples of more than 1-year-old bulls that did not show any clinical or macroscopic signs of papillomatosis, were obtained from a slaughterhouse operating in the province of Sivas, Türkiye.

2.2 | Collection of Samples and Histopathological Examination

Half of the testicular tissue samples of male cattle slaughtered at the slaughterhouse were fixed in a 10% buffered formalin solution for 24 h. In addition, the other half of the testicle samples were taken with a sterile scalpel and stored at −80°C until polymerase chain reaction (PCR) examinations were performed. The detected tissues were reduced and placed in tissue cassettes. The tissues taken into the cassettes were washed in running tap water for 8 h, and the fixation solution was removed. They were then subjected to routine follow-up procedures and embedded in paraffin blocks. Sections of 5 μm from each paraffin tissue block were placed on normal slides for histopathological examination and polylysine slides for immunohistochemical and immunofluorescence examination. To remove paraffin from the tissue sections placed on normal slides, they were kept in an oven at 58°C for 3 h. At the end of this period, these slides were passed through sequential alcohol and xylol series. Afterwards, they were subjected to haematoxylin and eosin dyes, passed through sequential alcohol and xylol series and sealed with Entellan. The preparations stained with haematoxylin–eosin were examined and photographed under a light microscope (Zeiss AxioCam ERc 5s, Jena, Germany) at 10× and 40× magnifications (Akcakavak, Kazak, and Deveci 2023; Kazak et al. 2024; Akcakavak et al. 2024).

2.3 | Immunohistochemical Examination

Sections obtained from the blocks onto 5-μm-thick polylysine slides were kept in the oven (60°C) for 1 h and then deparaffinised by passing through the xylene and alcohol series. The sections were rehydrated in distilled water for 5 min, kept in 3% H_2O_2 for 10 min to inactivate endogenous peroxidase and then washed twice with phosphate-buffered saline (PBS). To reveal the antigen in the tissues, it was treated with antigen retrieval solution (BioShop, EDTA, Catalogue no. EDT001, 0.037%, pH 8.0) in a microwave oven at 600 W for 10 min. To prevent non-specific binding, a protein-blocking solution (Catalogue no. TA-125-UB; Thermo Fisher) was added, left for 10 min and washed once with PBS. Then, the sections were incubated overnight at 4°C with a 1/100 dilution of monoclonal mouse anti-BPV (Catalogue no. MBS320197; MyBioSource), a 1/100 dilution of polyclonal rabbit anti-γH2AX antibody (Catalogue no. NB100-2280; NovusBio) and a 1/100 dilution of monoclonal mouse anti-cytochrome c antibody (Catalogue no. ab110325; Abcam). The sections washed with PBS were treated as recommended in the Lab Vision UltraVision Large Volume Detection System: anti-Polyvalent, HRP secondary immunohistochemistry kit (Catalogue no. TP-125-HL; Thermo Fisher). 3.3′-Diaminobenzidine was used as a chromogen to visualise antigen-antibody binding. After counterstaining with Mayer's haematoxylin, the sections were passed through the alcohol xylene series again, covered with Entellan, examined under a light microscope (Zeiss AxioCam ERc 5s, Jena, Germany) at 10× and 40× magnifications and photographed (Akcakavak, Kazak, and Deveci 2023; Kazak et al. 2024; Akcakavak et al. 2024).

2.4 | Immunofluorescence Examination

For this purpose, 5-μm sections placed on polylysine slides were first kept in an oven for 1 h to remove paraffin. Then, they were passed through xylene and alcohol series and kept in distilled water for 5 min. To reveal the antigen in the tissues, they were treated with antigen retrieval solution (BioShop, EDTA, Catalogue no. EDT001, 0.037%, pH 8.0) in a microwave oven at 600 W for 10 min. To prevent non-specific binding, a protein-blocking solution (Catalogue no. TA-125-UB; Thermo Fisher) was dropped, left for 10 min and washed once with PBS. Washed sections were treated with monoclonal mouse anti-BPV antibody (BPV, Catalogue no. MBS320197, 1/100 dilution; MyBioSource), polyclonal rabbit anti-γH2AX antibody (Catalogue no. NB100-2280; NovusBio) 1/100 and monoclonal mouse anti-cytochrome c antibody (Catalogue no. ab110325, 1/100 dilution; Abcam) and incubated overnight at +4°C. After washing the tissues once again with PBS, goat anti-mouse IgG FITC (Catalogue no. 115-095-003; Jackson Immunoresearch) secondary antibody for BPV and

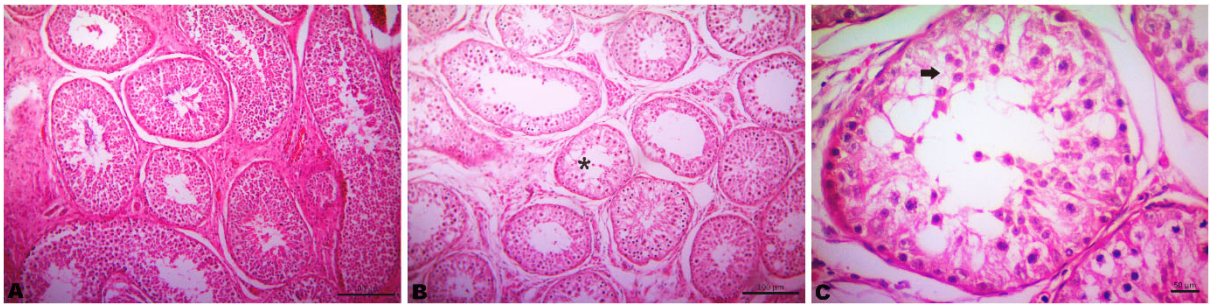

FIGURE 1 | (A) Histological appearance of healthy testicular tissue. (B) Histopathological appearance of testicular tissue determined to be BPV immunopositive by IHC and IF methods. Degenerative seminiferous tubule (*). (C) Histopathological appearance of testicular tissue determined to be BPV immunopositive by IHC and IF methods. Degenerative seminiferous tubule cells (arrow).

cytochrome c antibodies and goat anti-rabbit IgG FITC (Jackson Immunoresearch) for γH2AX primary antibody. The secondary antibody (Catalogue no. 111-095-003) was diluted 1/100, treated in a dark environment for 60 min and washed with distilled water. Then, 4′,6-diamidino-2-phenylindole dihydrochloride was dropped and examined under a fluorescent microscope (Zeiss Axio Vert.A1, Jena, Germany) at 40× magnification and photographed (Karakurt et al. 2021).

2.5 | PCR Method for BPV

Testicular tissue samples stored at −80°C were brought to room temperature and samples weighing 10 mg were taken into 1.5 mL sterile Eppendorf tubes. Three hundred microlitres of K buffer (20 mM Tris, 150 mM NaCl, 10 mM EDTA, 0.2% sodium dodecyl sulfate) and sterile distilled water were added. Five microlitres of 20 mg/mL Proteinase K was added to each tube. After the samples were vortexed, they were incubated in a bain-marie at 56°C for an average of 2 h. After incubation, they were subjected to boiling treatment for 10 min for proteinase K inactivation. After this stage, phenol extraction was applied to save the DNA from protein residues.

For this purpose, an equal volume of 25:24:1, phenol:chloroform:isoamyl alcohol was added to the tubes and thoroughly shaken by hand for 5 min. Subsequently, they were centrifuged at 13,000 rpm and the upper phase was carefully collected and transferred to newly identified tubes. 2–2.5 times the collected amount of pure ethyl alcohol stored at −20°C and 1/10 of 3 M Na acetate were added. After this process, the samples were vortexed and precipitated at −20°C for 2 h. Afterwards, the samples were vortexed at 13,000 rpm for 10 min. Right after the liquid decanted was added 300 μL of 70% concentration ethyl alcohol and centrifuged at 13,000 rpm for 5 min. After centrifugation, the liquid was carefully discarded, and the tubes were left to dry by turning them upside down on drying paper for approximately 10 min. After drying, 100 μL of sterile distilled water was added to each tube and vortexed. After the resulting mixture was subjected to purity and quantity control in the nanodrop device, it was ready to be used as target DNA in PCR. PCR samples were prepared as 20 μL in total for each DNA sample: Quick-Load Taq 2X Master Mix (Catalogue no. M0271L, 12.5 μL; NEB), 10 μM forward primer (0.4 μL), 10 μM reverse primer (0.4 μL), DNA extract (100 ng) and completed to 20 μL with nuclease-free ddH_2O. DNA samples were amplified using PCR primer pairs: MY11 (forward; 59-GCMCAGGGWCATAAYAATGG-39) and MY09 (reverse; 59-CGTCCMARRGGAWACTGATC-39). The previously confirmed BPV-positive sample was provided by the Virology Department of the Faculty of Veterinary Medicine at Kafkas University. After a preliminary denaturation at 95°C for 5 min, PCR was performed as 40 cycles of 30 s of denaturation at 95°C, 30 s of primer annealing at 51°C and 1 min of primer extension at 72°C, followed by a final extension of 10 min at 72°C. Five microlitres of the amplified material were analysed by electrophoresis for 35 min on a 1.5% agarose gel in TBE buffer by adding ethidium bromide (1 μg/mL). Bands specific to BPV DNA were identified by size determination under UV light.

3 | Results

3.1 | Microscopic Findings

In the 100 testicular tissues examined, samples determined to be BPV immunopositive by IHC and IF methods and samples determined to be BPV immunonegative were examined histopathologically. As a result of the examinations, degenerative tubules were found in some samples determined to be both BPV positive and BPV negative (Figure 1).

3.2 | Immunohistochemical Findings

As a result of immunohistochemical examinations, six (6%) BPV immunopositivity was detected. BPV immunopositivity was found in spermatogonia, spermatocyte, spermatid and Sertoli cells of testicular tissue. While BPV immunopositivity was observed in spermatogonia cells in all six samples in which immunopositivity was detected, it was observed in spermatocytes in four, in spermatids in three and in Sertoli cells in four (Table 1). It was determined that the immunopositivities were located intracytoplasmically (Figure 2).

As a result of immunohistochemical examinations, γH2AX immunopositivity was found in two of six samples (33.3%) determined BPV positive. Immunopositivity was observed in the spermatid cells of the testicular tissue (Table 2). Immunopositivities were found to be intranuclear (Figure 3). As a result

TABLE 1 | Distribution of BPV immunopositivity at the cellular level.

BPV positive samples	Spermatogonium	Spermatocyte	Spermatid	Sertoli
1	+	+	−	+
2	+	−	+	−
3	+	−	−	−
4	+	+	+	+
5	+	+	−	+
6	+	+	+	+

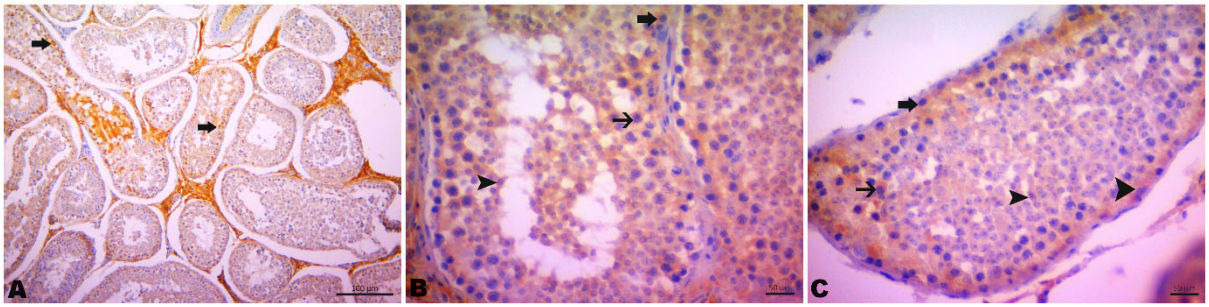

FIGURE 2 | (A) BPV immunopositivity in seminiferous tubules (arrows). (B) BPV immunopositivity in spermatogonium (arrow), spermatocyte (thin arrow) and spermatid cells (arrowhead). (C) BPV immunopositivity in spermatogonia (arrow), spermatocyte (thin arrow), spermatid (arrowhead) and Sertoli cells (large arrowhead).

TABLE 2 | Distribution of γH2AX and cytochrome c immunopositivity positivity at the cellular level.

BPV positive samples (IHC)	γH2AX				Cytochrome c			
	A	B	C	D	A	B	C	D
1								
2	−	−	+	−	+	+	+	−
3	−	−	−	−	+	+	−	+
4	−	−	−	−	+	−	−	−
5	−	−	+	−	+	−	−	+
6	−	−	−	−	+	+	−	−

Note: A, spermatogonium; B, spermatocyte; C, spermatid; D, Sertoli.

of immunohistochemical examinations, it was observed that all samples with BPV immunopositivity (100%) also had cytochrome c immunopositivity. It was found in spermatogonia in six samples with BPV immunopositivity, in spermatocytes in four, in spermatids in two and in Sertoli cells in three (Table 2). It was determined that the immunopositivities were located intracytoplasmically (Figure 3). Mild cytochrome c immunopositivity was also detected in samples without BPV immunopositivity (Figure 3).

3.3 | Immunofluorescence Staining Findings

As a result of immunofluorescence examination of testicular tissues, BPV immunopositivity was detected in 11 of 100 (11%) of bovine testicular tissues. Positivity was found in spermatogonia in 11 of these testicular samples, in spermatocytes in 9, in spermatids in 3 and in Sertoli cells in 5 (Table 3; Figure 4). As a result of the immunofluorescence examination of the 100 testicular tissue samples evaluated, γH2AX immunopositivity was found in 4 of the 11 samples with BPV immunopositivity. While positivity was seen in spermatid cells in four of the samples, mostly in parallel with the immunohistochemical findings, it was found in spermatogonia in one sample, in spermatocytes in two and in Sertoli cells in two (Table 4; Figure 4). Cytochrome c immunopositivity was found in spermatogonia, spermatocyte, spermatid and Sertoli cells of 11 samples determined BPV immunopositive. The presence of immunopositivity was observed in spermatogonia in all 11 samples that were determined to be positive, in spermatocytes in 7, in spermatids in 3 and in Sertoli cells in 5 (Table 4; Figure 4).

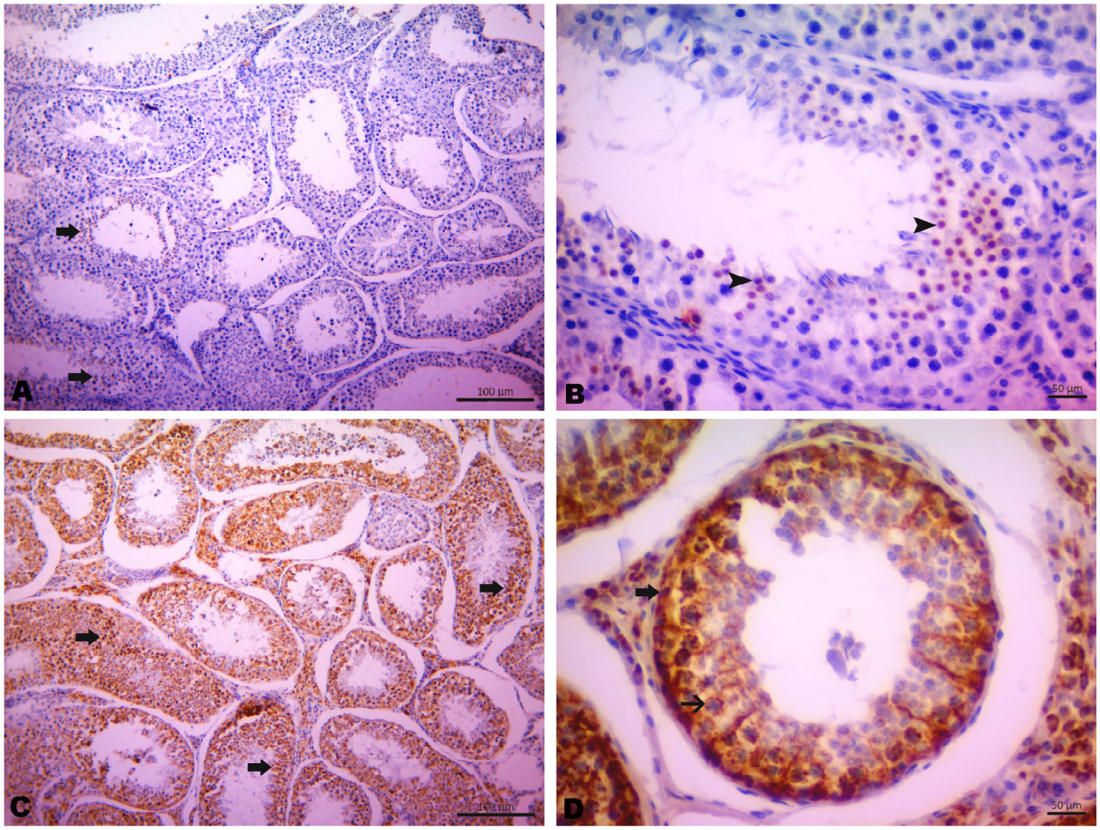

FIGURE 3 | (A) γH2AX immunopositivity in the seminiferous tubules (arrows) in the BPV immunopositive sample. (B) γH2AX immunopositivities (arrowhead) in spermatid cells in the BPV immunopositive sample. (C) Cytochrome c immunopositivity in the seminiferous tubules (arrows) in the BPV immunopositive sample. (D) Cytochrome c immunopositivity in spermatogonia (arrow) and spermatocyte cells (thin arrow) in BPV immunopositive samples.

TABLE 3 | Distribution of BPV immunofluorescence positivity at the cellular level.

BPV positive samples	Spermatogonium	Spermatocyte	Spermatid	Sertoli
1	+	+	+	+
2	+	+	−	+
3	+	+	−	−
4	+	+	−	−
5	+	−	−	+
6	+	+	−	−
7	+	+	+	+
8	+	−	−	−
9	+	+	−	−
10	+	+	−	+
11	+	+	+	−

3.4 | PCR Findings

PCR method was applied to 100 testicular tissue samples using PCR primers. By evaluating the gel electrophoresis images obtained, it was determined that four (4%) samples were positive (Table 5; Figure 5).

4 | Discussion

Papillomas or warts caused by papillomaviruses, known as epitheliotropic, are seen mostly on the scalp, tongue, udders, penis, vulva, oral cavity and upper digestive tract in cattle (Rector and Van Ranst 2013; Timurkan and Alcigir 2017). BPV causes

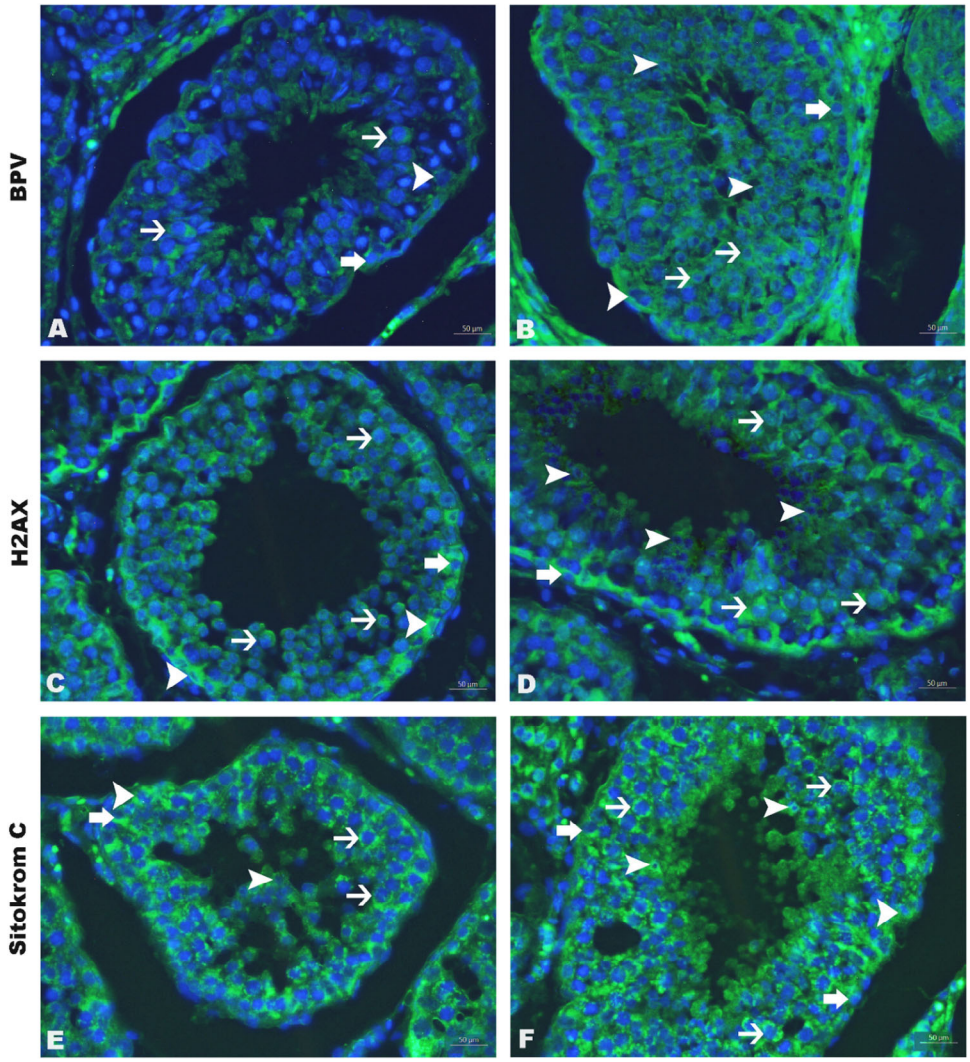

FIGURE 4 | (A) BPV immunopositivity in spermatogonia (arrow), spermatocytes (thin arrow) and Sertoli cells (large arrowhead). (B) BPV immunopositivity in spermatogonium (arrow), spermatocyte (thin arrow), spermatid (arrowheads) and Sertoli cells (large arrowhead). (C) γH2AX immunopositivity in spermatogonia (arrow), spermatocytes (thin arrow) and Sertoli cells (large arrowhead) in the BPV immunopositive sample. (D) γH2AX immunopositivity in spermatogonia (arrow), spermatocyte (thin arrow) and spermatid cells (arrowhead) in the BPV immunopositive sample. (E) Cytochrome c immunopositivity in spermatogonia (arrow), spermatocyte (thin arrow), spermatid (arrowhead) and Sertoli cells (large arrowhead) in BPV immunopositive sample. (F) Cytochrome c immunopositivity in spermatogonia (arrow), spermatocyte (thin arrow), spermatid (arrowhead) and Sertoli cells (large arrowhead) in BPV immunopositive sample.

benign tumours such as cutaneous papilloma and fibropapilloma in cattle or malignant tumours such as bladder and oesophageal cancers, causing significant economic losses such as growth retardation, weight loss and decreased milk production (Munday 2014; Rojas-Anaya et al. 2016). The disease caused by BPV, known as bovine papillomatosis, is a benign tumour generally characterised by the development of multiple papillomas (Munday 2014; Beytut 2017). Especially in experimentally induced infections, tumour lesions appeared between 30 and 59 days and showed spontaneous regression within 1–14 months (Sundberg and Reichmann 1993). However, it has been stated that it is more common in cattle under 2 years of age that are kept in close contact with each other (Olson et al. 1969). In addition, it has been suggested that animals that are 2 years of age and older are more resistant to the agent than younger animals, possibly because they have been previously exposed to the virus. In the presented study, it was preferred to investigate the presence of the BPV agent in testicular tissue samples obtained from cattle over 1-year old, belonging to different herds, that did not show macroscopic papilloma findings, slaughtered in a slaughterhouse in the province of Sivas.

In studies conducted on the distribution of the agent in the body, other than skin lesions, the location of the agent is oocytes, ovary, uterus, cumulus cells, uterine fluids, semen and spermatozoa have been demonstrated to be present in the reproductive system (de Carvalho et al. 2003; Yaguiu et al. 2017; Silva et al. 2013). When these studies were examined, de Carvalho et al. (2003) in Brazil, after the slaughtering process of two female cattle selected from a herd where papillomatosis was commonly observed but which did not show macroscopic signs of papillomatosis within the herd, by extracting DNA from uterine tissue, ovary, oocyte and cumulus cell samples, the presence of BPV was demonstrated in female reproductive cells by PCR method. Similarly, Yaguiu et al.

TABLE 4 | Distribution of γH2AX and cytochrome c immunofluorescence positivity at the cellular level.

BPV positive samples (IF)	γH2AX				Cytochrome c			
	A	B	C	D	A	B	C	D
1	−	−	+	+	+	+	−	+
2	−	−	−	−	+	−	−	−
3	−	−	−	−	+	+	+	+
4	−	−	+	−	+	+	−	−
5	−	−	−	−	+	−	−	−
6	−	+	+	−	+	+	+	+
7	−	−	−	−	+	+	+	−
8	+	+	+	+	+	−	−	+
9	−	−	−	−	+	+	−	+
10	−	−	−	−	+	−	−	−
11	−	−	−	−	+	+	−	−

Note: A, spermatogonium; B, spermatocyte; C, spermatid; D, Sertoli.

TABLE 5 | PCR well contents.

Well number	1	2	3	4	5	6	7	8	9	10	11	12
Well content	L	PK	+	−	−	+	−	+	+	−	−	−
Size of positive samples in bp		450 bp	450 bp			450 bp		450bp	450bp			

Abbreviations: L, ladder; PK, positive control.

(2017) collected in Brazil samples of cutaneous papilloma, ovary, uterus and semen from male and female cattle, and in the study conducted on these samples, unlike the study conducted by de Carvalho et al. (2003), BPV positivity was also detected in semen samples. Although the localisation of BPV in the reproductive system has been reported, as in these studies, the fact that no study has been found in the literature regarding the presence of the virus in testicular tissue has been the main subject of our research. As a result of our study, the BPV factor positivity was determined as 4 by the PCR method, 6 by the IHC method and 11 by the IF method, indicating that the agent was also present in the testicles.

Today, virus isolation, PCR, Western blot, comet assay, in situ hybridisation, tissue fluorescent antibody technique and IHC staining methods are used in the diagnosis of BPV (Beytut 2017; Yaguiu et al. 2017; Santos et al. 2021; Karakurt 2021). In the presented study, BPV positivity was evaluated from 100 testicular samples collected by PCR, IHC and IF staining methods. PCR has been stated as the most reliable among these methods (Cutarelli et al. 2021; De Falco et al. 2021). While worldwide and in our country (Türkiye), there are many studies on the determination of BPV from tissues and body fluids with the PCR method (Timurkan and Alcigir 2017; Yaguiu et al. 2017; Carvalho et al. 2013; Alçiğir and Timurkan 2018), no studies have been found using samples obtained directly from testicular tissue. Yaguiu et al. (2017), in their study, using the PCR method on 14 samples containing skin papilloma, uterus, ovary and semen, detected BPV positivity in 6 skin papilloma samples, 3 uterus samples, 1 ovarian sample and 2 semen samples. Pathania et al. (2012) reported that in their study using the PCR method on 24 urine and 120 bladder samples, both with and without clinical symptoms, they found a positivity rate of 50% in the urine samples and 68.6% in the bladder samples. Dagalp et al. (2017), in their study on tissue samples, found BPV positivity in 31 of 35 samples (88.6%). In the presented study, it was thought that the lower positive results obtained with the PCR method compared to the IHC and IF methods were due to standardisation problems occurring in the optimisation steps of the PCR method.

In studies conducted worldwide, there are many detection and prevalence studies on papillomaviruses, a common and well-known old virus family (Carvalho et al. 2013; Hamad, Al-Shammari, and Odisho 2016; Grindatto et al. 2015; Bocaneti et al. 2016; Daudt et al. 2018). Araldi et al. (2015) used skin materials collected from 6 cattle without any signs of cutaneous papilloma and 18 cattle with cutaneous papilloma and showed by PCR method that 17 of 24 samples (70.8%) were BPV positive. Carvalho et al. (2013) examined 72 skin samples collected from two different herds with a high rate of papillomatosis and found that 64 samples (89%) were BPV positive. Grindatto et al. (2015), in their study, as a result of histopathological and PCR analyses of papilloma-like samples collected from 70 cattle and 1 donkey, it was revealed histopathologically that all samples were papilloma. BPV positivity was detected in all samples using the PCR method parallel to histopathological analyses. In addition,

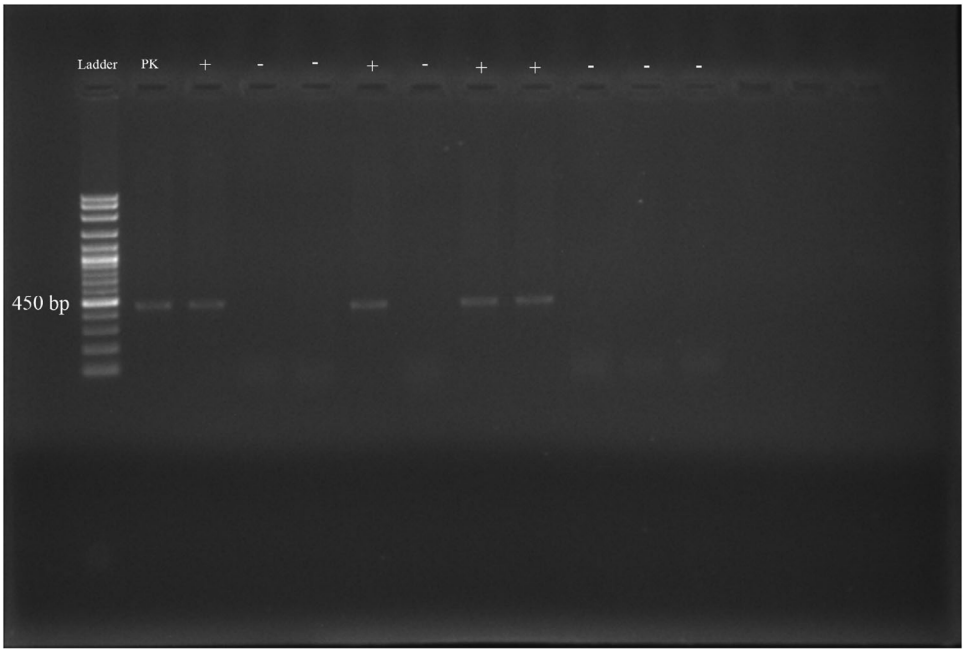

FIGURE 5 | PCR analysis findings. First well ladder. Well 2 is positive control. BPV positive samples (450 bp) in wells 3, 6, 8 and 9. BPV negative samples in wells 4, 5, 7, 10, 11 and 12.

there are some previous studies on the prevalence of BPV in our country (Türkiye) (Timurkan and Alcigir 2017; Beytut 2017; Alçiğir and Timurkan 2018). In the study conducted by Timurkan and Alcigir (2017), macroscopic papilloma samples were collected from Ankara, Bursa and Samsun regions, and the 29 collected samples were evaluated histopathologically and virologically. As a result of the evaluation, the PCR method revealed that 11 of 29 samples (37.9%) were BPV positive. Again, in a study conducted by Beytut (2017) in the region of Kars in our country (Türkiye), skin and teat papillomas of 450 cattle of various breeds, ages and genders slaughtered in a local slaughterhouse were examined. While proliferative lesions were observed on the skin in 19 of the cattle included in the study and on the teat in 12, hard masses were found in the genital area in three of them, and these structures were evaluated histopathologically and immunohistochemically. As a result of the histopathological examination of these 34 samples, fibropapilloma, 9 papilloma and 3 fibroma were detected in 10 of the samples. BPV immunopositivity was found in 16 (44.4%) samples in immunohistochemical examinations. In addition, Ataseven, Kanat, and Ergün (2016) tissue samples from 23 cattle showing macroscopic papilloma findings collected from Hatay, Adana and Osmaniye provinces and blood samples from nine cattle without any macroscopic findings were examined by PCR method. The presence of BPV was detected in 16 of 23 tissue samples (69.6%) and in all 9 blood samples (100%). Studies show that not all papilloma/tumour-like structures seen macroscopically are papillomas. In addition, it appears that the BPV agent may not be present in all tissues that histopathologically are papillomas.

In the presented study, in addition to the presence of BPV, the relationship between the virus and γH2AX and the virus and cytochrome c were examined. Some studies have been conducted on the relationship of papillomaviruses with γH2AX (Sakakibara, Mitra, and McBride 2011; Fradet-Turcotte, Bergeron-Labrecque, and Moody 2011). Sakakibara, Mitra, and McBride (2011), in their study on E1 and E2 proteins, which are necessary for the viral genome replication of papillomaviruses in cell culture, found that the simultaneous expression of E1 and E2 proteins caused the suppression of the growth of host cells. They identified the E1 protein as the main cause of growth suppression and observed that this situation was accompanied by DNA damage activation resulting in γH2AX phosphorylation, ATM (Ataxia-Telangiectasia Mutated) and Chk2 (Checkpoint Kinase 2). They revealed by the IF method the presence of γH2AX expressions. In addition, in the cell culture study conducted by Fradet-Turcotte et al. (2011), they showed by the IF method that during the activation of the E1 protein, which enables the replication of papillomaviruses in the host cell, it causes DNA damage by inducing γH2AX phosphorylation in the host tissue cells, and that γH2AX phosphorylation is intensely observed in the nuclei of the cells where the expression of the E1 protein is found. In the presented study, the presence of γH2AX expression in testicular tissue was investigated by the IHC and IF methods, and γH2AX immunopositivity was determined in two samples by the IHC method and in four samples by the IF method. This has been interpreted as implying that the virus may cause double DNA breaks.

Cytochrome c oxidase is the last enzyme of the mitochondrial electron transport chain that activates the oxidative phosphorylation process, where the majority of ATP production occurs (Kalpage et al. 2020). Various studies have been conducted on the relationship between papillomaviruses and cytochrome c (Savini et al. 2020; Padilla, Leung, and Carson 2002). Regarding this, Savini et al. (2020) obtained cytochrome c positivity in their study using the PCR method in a papilloma sample taken from the mammary tissue of a sheep. Padilla, Leung, and Carson (2002), in a study investigating the effects of chemotherapy on cases of papillomavirus-induced cervical cancer, revealed the

presence of cytochrome c release, a marker of mitochondrial damage, by the IF method in cell culture. In addition, Jung et al. (2017), in their study investigating the relationship between papillomavirus in oropharyngeal head and neck cancers, stated that there was cytochrome c release in all the cell lines studied. In the presented study, cytochrome c release was observed in all 100 samples examined by IHC and IF methods. The presence of immunopositivity in all samples was interpreted as being due to another reason, yet, the high level of cytochrome c release in BPV-positive samples suggested that cytochrome c expression had been induced by the virus.

There are some limitations to our study. Our major limitation is that the agent (BPV) primary was not subtyped. Future studies can provide a more comprehensive perspective by performing this subtype.

5 | Conclusion

In the presented study, positivity was found in 6 samples in immunohistochemical staining, 11 in immunofluorescence staining and 4 samples in PCR method, which were performed to reveal the presence of BPV agent in the testicular tissues of male cattle that did not have any macroscopic findings of papilloma in any part of their body. This study confirms the presence of BPV in testicular tissues of apparently healthy bulls, suggesting that semen could act as an additional source of virus transmission. Moreover, our results suggested that BPV caused double-stranded DNA breaks and increased cytochrome c expression.

Author Contributions

Ozhan Karatas: conceptualisation, methodology, validation, formal analysis. **Mustafa Ozkaraca**: investigation, conceptualisation, validation, formal analysis. **Mustafa O. Atasoy**: methodology, validation, visualisation.

Ethics Statement

The study was approved by the Unit Ethics Committee of Atatürk University Faculty of Veterinary Medicine (2019/13).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data of the relevant study can be obtained from the corresponding author.

Peer Review

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