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RESEARCH ARTICLE

A Histopathological, Immunohistochemical and Molecular Study of Cutaneous Bovine Papillomatosis

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Summary

In animals and man, some forms of cutaneous papillomas are caused by papillomaviruses. Twelve different genotypes of bovine papillomavirus (BPV) have been identified. BPV-1 through BPV-12 are all strictly species-specific. BPV-1 and BPV-2 are associated with fibropapillomas in cattle; these tumors are formed by excessive proliferation of virus-infected dermal fibroblasts and epidermal keratinocytes. In the present study well known diagnostic procedures including histopathological, immunohistochemical and molecular methods were used for the diagnosis of bovine papillomavirus infection.Samples were taken randomly from five cattle with multiple cutaneous tumor formations for the diagnosis of bovine papillomavirus infection occurring commonly in herds.In the present study five cattle were examined for the presence of multiple cutaneous tumors. Cutaneous fibropapillomatosis were confirmed histopathologically and immunohistochemically. BPV-1 and BPV-2 which are etiological agents for the bovine cutaneous fibropapillomatosis were investigated by PCR. BPV serotyping was performed from five cattle all coming from three different farms. In conclusion papillomaviral DNA was detected by the PCR. The amplified long control region (LCR) DNA sequence was identical to that of BPV-1 and negative for the BPV-2 DNA. When considered economical impact of the disease associated with the abundance of the infection significance of protective measures and vaccine studies come forward.

Keywords: *Bovine papillomavirus type 1/2 (BPV-1/2), Fibropapilloma, Cattle*

Sığır Deri Papillomatozisinin Histopatolojik, İmmunohistokimyasal ve Moleküler Araştırılması

Özet

Türe özgü 12 farklı genotipi tanımlanmış sığır papillomavirusları (BPV), hayvanlar ve insanlarda deri papillomlarının bazı formlarına neden olurlar. Sığırlarda BPV-1 ve BPV-2 ile enfekte dermal fibroblast ve epidermal keratonositlerin aşırı çoğalması sonucunda fibropapillomlar oluşmaktadır. Bu çalışmada sığırların deri tümörlerinde etiyolojik ajanlardan biri olan papilloma viruslarının rolü araştırılmıştır. Papillomların yaygın olarak bulunduğu sürülerde, BPV enfeksiyonunun teşhisi amacıyla; rasgele seçilen multiple deri tümörleri bulunan beş sığırdan örnekler alındı. Araştırmada papilloma enfeksiyonunun teşhisine yönelik olarak dünyada uygulanırlığı kabul edilen histopatolojik, immunohistokimyasal ve moleküler yöntemler kullanıldı. Histopatolojik ve immunohistokimyasal metotlarla deri fibropapillomatozisleri doğrulandıktan sonra, sığır deri fibropapillomatozislerine neden olan BPV-1 ve BPV-2 nin rolü, üç çiftlikten alınan beş sığır örneğinde moleküler olarak PZR (polimeraz zincir reaksiyonu) yöntemi ile araştırıldı. Sonuçta PZR metodu ile BPV-1'in DNA zincirindeki kontrol bölgesi tespit edilmesine rağmen BPV-2 negatif bulundu. Etiyolojik nedeni ortaya konan bu araştırma ile enfeksiyonun yaygınlığına bağlı ekonomik girdiler değerlendirildiğinde çalışma sonucunda, aşı çalışmalarının ve koruyucu tedbirlerin önemi ön plana çıkmaktadır.

Anahtar sözcükler: *Bovine papillomavirus tip 1/2 (BPV-1/2), Fibropapillom, Sığır*

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INTRODUCTION

Cutaneous papillomatosis is a viral disease caused by the papillomavirus and is characterized by epithelial hyperplasia and proliferation of connective tissue. Pathological changes similar to warts appear on the head, neck and other areas of the body. They do not generally pose a clinical problem, but they can sometimes become malignant when accompanied by certain genetic and environmental factors. The infection can result in significant economic losses in animal husbandry due lower milk and meat yields and reduced hide quality. Even though it can appear on cattle of any age, it is seen more commonly and is more severe in animals less than two years old [1-3].

The primary source and natural carrier of the virus is cattle. The contagion enters the body through scratches or other defects. The infection occurs through both direct and indirect contact. Other factors that play a significant role in the occurrence of the disease are contaminated materials, milking machines, tuberculosis injections, malnutrition, and hormonal imbalances as well as semen, mutations and long-term exposure to sunlight if there is immunodeficiency [4-6].

Bovine papillomaviruses (BPV) are non-enveloped, icosahedral symmetrical DNA viruses 50-55 nm in diameter from the papovavirus family (papilloma, polioma, vacuolating viruses). To date, 12 different species-specific serotypes of the virus have been reported. BPV-1 and BPV-2 are fibro-papillomaviruses related to the genus Deltapapillomavirus that show an affinity for epithelial and dermis tissue [7-10]. BPV-3, BPV-4, BPV-6, BPV-9 and BPV-10 are epitheliotropic and belong to the genus Xipapillomavirus. BPV-5, BPV-7 and BPV-8, on the other hand, belong to the genus Epsilon-papillomavirus and cause both epithelial papillomas and fibropapillomas in the skin [11]. Under natural conditions, papilloma viruses are specific to the host, but BPV-1 and BPV-2 also infect horses, causing fibroblastic tumors [12].

Diagnosis of infection is based on clinical symptoms, histo-pathological findings and the use of an electron microscope [13]. Another important aspect of virus identification is polymerase chain reaction (PCR). In recent years, there have been numerous studies conducted with FAP59/FAP64 and MY09/MY11 consensus primers designed based on the regions of the genome that code the structural proteins L1, L2, E6 and E7 [14-17]. In the beginning, consensus primers designed based on the region of the genome that codes L1 (HPV) in the human papilloma virus were commonly used to identify the papillomavirus in humans, cattle and other animals [6,14-16,18].

The purpose of this study was to conduct histo-pathological, immunohistochemical and molecular research on cutaneous papillomatosis in cattle that showed clinical symptoms of being infected with the papillomavirus and to discuss recommendations for controlling this infection.

MATERIAL and METHODS

Animals and Sample Collection

In this study, five cattle from three different herds were examined for the presence of BPV. The cattle showed multiple, cutaneous tumors localized in the head, neck, back and muzzle regions.

The samples were obtained after cleaning the area with water and soap and decontamination with 70% ethanol. Segments of warts were removed by parallel incision in the surface of the skin using a disposable sterile scalpel and kept in sterile tube *(Fig. 1)*.

Fig1. Macroscopic view of papillomas
Şekil 1. Papillomların makroskobik görünümü

Histopathology

For histopathological examinations, gross lesions were recorded and tissue specimens were collected from skin lesions, fixed in formaldehide and embedded in paraffin wax, sectioned at 5 µm and stained with Lillie Mayer Haematoxylin-Eosin (H&E).

Immunohistochemistry

Sections (5 µm) from the samples were labelled immuno-histochemically by the streptavidin-biotin-peroxidase complex (ABC) technique for detection of the papilloma virus. Serial sections were dewaxed in xylene and hydrated through graded alcohols. Endogenous peroxidase activity was blocked with hydrogen peroxide 3% in methanol for 15 min. The sections were rinsed with phosphate buffered saline (PBS, pH 7.2) and subsequently placed into citrate buffer (pH 6.0) in a microwave oven (800 W) for 10 min for antigen retrieval. After washing with PBS the sections were then incubated with rabbit anti papilloma virus antibody (Dako Cytomation, Glostrup, Denmark) for 30 min. Following brief rinsing with PBS the sections were incubated with biotinylated secondary antibody (Histostain®-Plus kit, Cat. No. AA85-9043; Invitrogen corp, Camarillo, CA, USA) and Streptavidin HRP (Histostain®-Plus kit) each overlaid onto the sections for 10 min at RT. Papilloma virus positive cells

were visualized using AEC and counterstained with Gill's haematoxylin. Primary antibodies were omitted from negative control sections, which were incubated with either TBS or diluted normal serum from the species in which the primary antibody was raised.

Polymerase Chain Reaction Amplification of Viral DNA

Total DNA was extracted from 300 µl of samples using the phenol–chloroform method [19]. These extracts were tested by polymerase chain reaction (PCR) using primers specific to BPV1 (forward: 5′-ggagcgcctgctaactatagga-3′ and reverse: 5′-atctgttgtttgggtggtgac-3′), BPV2 (forward: 5′-gttataccacccaaagaagaccct-3′ and reverse: 5′-ctggttgca acagctctctttctc-3′), as described by Lindsey et al.[6] These primers are complementary to the L2 and L1 regions of BPV1 and BPV2 genes, respectively. The expected product sizes were 301 and 164 bp. Cycling conditions were as follows: 1 cycle of 96°C for 6 min followed by 40 cycles of 95°C for 30 sec, 72°C for 1 min, 56°C for 45 sec, and a final extension of 72°C for 10 min. The amplicons visualized by 1% ethidium-bromide-stained agarose gel.

RESULTS

Histopathological Analysis

Histopathological investigation of samples revealed a marked ortho or parakeratotic hyperkeratosis. The dermal papillae were elongated with irregular rete ridge formation covered by acanthotic epidermis *(Fig. 2)*. In the prickle cell layer, single cells or small groups of cells with vacuolated cytoplasm were seen *(Fig. 3)*. Others showed variable degrees of ballooning degeneration (koilocytes) with presence of abundant clumped, pleomorphic keratohyaline granules. Intranuclear inclusion bodies were variable findings.

In the dermal layer the proliferating cells were large, plumb

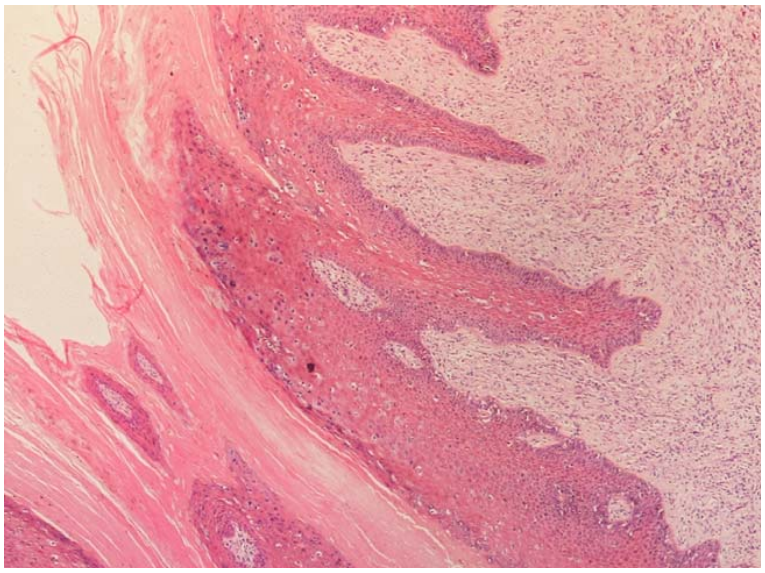

Fig 2. Papilloma with thickened, irregular epidermis covered by a layer of keratin H&E x4

Şekil 2. Kalınlaşmış papillomun, keratin bir tabaka ile kaplı düzensiz epidermisi H&E x4

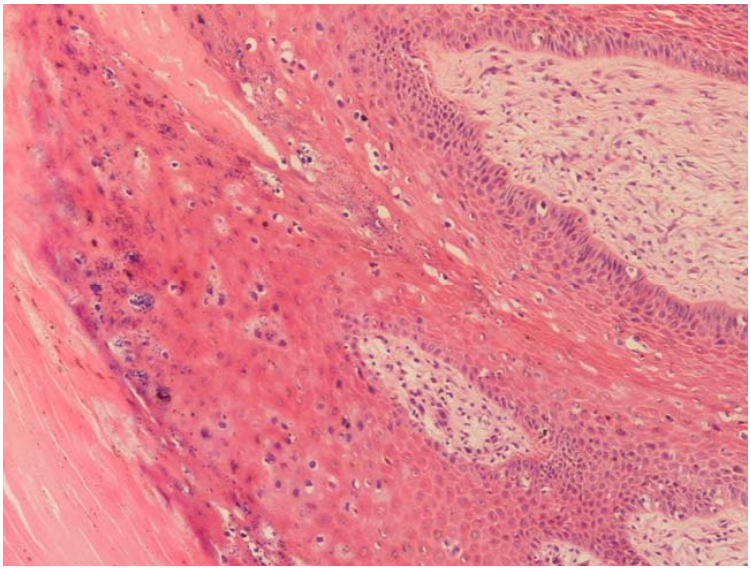

Fig 3. Hyperplasia and vacuolation of the stratum spinosum, bovine cutaneous papilloma H&E x10

Şekil 3. Sığır deri papillomasında; Hiperplazi ve stratum spinozumdaki hücrelerde vakuolizasyon H&E x10

fibroblasts arranged in haphazard whorls and fascicles. In others the connective tissue was mature, hyalinized and cell poor. In some cases epidermis was ulcerated and neutrophil margination was seen in the dilated and congested capillaries within the dermis. Neutrophilic exocytosis into the dermis and epidermis were seen in cases with secondary infection. In other cases the epidermal proliferation was minimal and characterised as slight acanthosis and accentuation of rete pegs. Occasionally melanin granules that were free within the subepidermal and within dermal melanophages.

Immunohistochemistry

Some nuclei in the granular and basal layers of the epidermis revealed intense positivity for papilloma virus antigen *(Fig. 4)*. In the stratum corneum most of the nuclear remnants exhibited strong positivity. This also occurred around small empty spaces, probably left by disappearing nuclei. In some cases, connective tissue of the fibropapillomas, some mesenchymal cells showed weak to intense positivity in the cytoplasm, sometimes, accompanied by positivity in the nuclei. Occasionally some vascular endothelial cells revealed weak immunolabelling. All skin structures were negative in the slides treated with TBS or normal serum instead of primary antibody.

Polymerase Chain Reaction

Wart samples collected from the five animals showed a 301 bp amplified product corresponding to the expected L2 gene fragment of BPV-1 segment, compared to the positive control. There was no specific BPV-2 DNA amplification as a result of PCR *(Fig. 5)*.

DISCUSSION

Diagnosis of cutaneous papillomatosis, which is commonly observed in cattle, is based on clinical symptoms, histopathological findings and the use of an electron microscope [13]. In this study, papilloma virus infection was confirmed by identifying BPV-1 nucleic acid with the PCR technique, clinical and immunohistochemical findings as well as histopathological findings.

The etiological agent for bovine cutaneous fibropapillomatosis is thought to be BPV-1 and BPV-2 [3,20]. Numerous PCR primers are used to identify papilloma viruses. Even though most of these primers have been designed using human papilloma viruses, they are also used to identify animal papilloma viruses, including bovine papilloma viruses [16-18,21]. The consensus primers FAP59/FAP64 and MY09/MY11 are commonly used to identify papilloma viruses in cattle and other animals [6,14,15,17].

After conducting the PCR procedure on full blood and lymphocyte cultures belonging to 54 six-year-old Holstein cows, Diniz et al.[22] positively identified BPV-1 in 74% (40/54) and BPV-2 nucleic acid in 87% (47/54) of the samples. Yanguiu et al.[23] and Freitas et al.[24] confirmed the existence of BPV-1

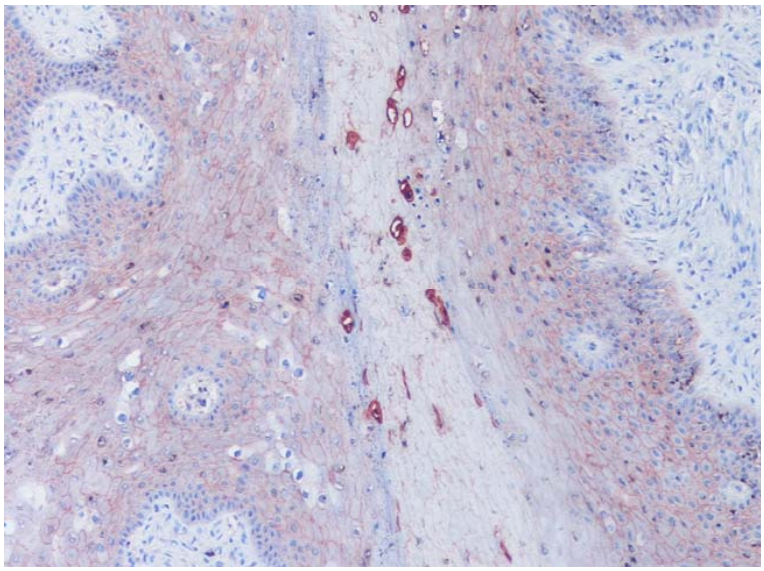

Fig 4. Nuclei in the epidermis are positive for papillomavirus antigen IHC. X10

Şekil 4. Epidermisteki hücre çekirdeklerinde papillomavirus pozitif reaksiyon IHC. X10

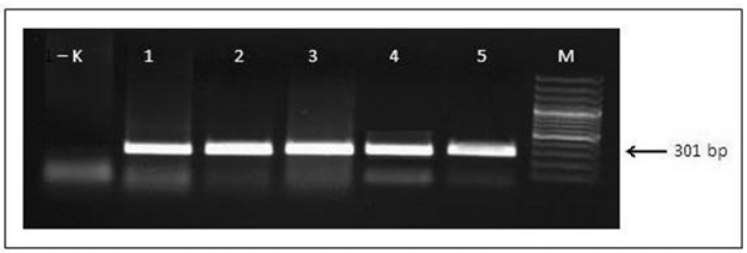

Fig 5. PCR amplification of viral DNA from papilloma samples. **M:** 100 pb DNA ladder; **K:** Negative control; **Line 1-5:** Samples used for the study

Şekil 5. PZR sonuçları. **M:** 100 bç DNA merdiveni; **K:** Negatif virus kontrol; **Hat 1-5 :** Araştırmada kullanılan örnekler

and BPV-2 nucleic acid with the PCR procedure in papilloma samples they collected from cattle clinically diagnosed with cutaneous papilloma.

Molecular research of the samples taken from papillomas generally found on the head and neck focused on BPV-1 and BPV-2 and were unable to obtain amplicons for BPV-2 that were as large as desired, but 301 base pairs of DNA product from the L1 region of BPV-1 was reproduced after the PCR technique was performed with consensus primer pairs. This shows us that BPV-1 is the primary active viral agent in bovine fibropapillomatosis.

Even though bovine papillomas are generally located on the head and neck, there have been reports that on some animals they also form in other areas of the body, such as the thorax [3,25-27], which substantiates the fact that in this study most of the lesions were located on the head and neck. The neoplasia were cauliflower-shaped with or without pedicles or resembled warts and some of them were bleeding on the surface or had erosive features.

Microscopic examination showed hyperkeratotic areas on the surface of the epidermis, hyperplasia in the keratinocytes in the stratum spinozum, vacuolic and balloon-like degeneration in the cells and a granular appearance in the keratinocytes. Free melanin granules were identified in the dermal and subepidermal melanophages. Epidermal acanthosis and hydropic changes were also observed in the keratinocytes of the epidermis.

Even though viral inclusions are reportedly rare in natural cutaneous papillomas [27-30], this study did identify intranuclear inclusion bodies. Immunohistochemical staining some nuclei in the granular and basal layers of the epidermis revealed intense positivity for papillomavirus antigen. In the stratum corneum most of the nuclear remnants exhibited strong positivity.

In conclusion BPV-1 was identified in the cattle as cause of bovine cutaneous fibropapillomatosis, but not BPV-2. It warrants more detailed study to confirm presence or absence of BPV-2 and other BPVs. Preventative measures should be taken in light of the ways in which the infection is spread, and prophylactic and therapeutic polyvalent vaccines should be used.

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