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Molecular identification of bovine papillomaviruses in dairy and beef cattle: first description of Xi- and *Epsilonpapillomavirus* in Turkey

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Abstract: In the current study, 23 papilloma/tumor-like samples obtained from cattle having clinical lesions and 9 blood samples collected from healthy-appearing cattle in Turkey were examined for bovine papillomavirus (BPV) DNA using the degenerate primers FAP59/64, and the different types of BPV were distinguished by type-specific primer sets. Furthermore, histopathological studies of papillomavirus were performed. A total of 7 BPV types (BPVs 1, 2, 3, 4, 6, 8, 9), including genera *Deltapapillomavirus*, *Xipapillomavirus*, and *Epsilonpapillomavirus*, were identified. In all samples, BPV-1 was the most common genotype (90.6%). Overall, coinfections were determined in 26 of the examined samples (81.3%), and coinfection with BPV-1/BPV-4/BPV-8 (21.9%) was the most frequently identified using BPV type-specific primers. Moreover, bovine leukemia virus, an oncogenic retrovirus, was detected from three cattle with tumor-like lesions (13.0%), which were also coinfected by different BPV types. Histopathologically, nine papilloma-like lesions were assessed and diagnosed as five fibropapillomas and four papillomas. BPV infection is an important cause of economic losses in the dairy and beef industry. Our study will be highly useful for guiding further large-scale epidemiological studies and providing detailed data on the risk factors associated with BPV infection in cattle populations in Turkey.

Key words: Bovine papillomavirus, cattle, histopathology, molecular identification, Turkey

1. Introduction

Bovine papillomaviruses (BPVs) belong to *Papillomaviridae* (1), a family of viruses that have oncogenic potential to cause benign and malignant tumors in association with proliferations of cutaneous or mucosal epithelia (2,3). Recently, bovine papillomaviruses have been classified into three genera: BPVs 1, 2, and 13 as *Deltapapillomavirus*; BPVs 5 and 8 as *Epsilonpapillomavirus*; and BPVs 3, 4, 6, 9, 10, 11, and 12 as *Xipapillomavirus*. BPV-7 has not yet been assigned to a genus (1,4). BPV-1 and BPV-2 have also been identified from equine sarcoids in horses as a result of interspecies transmission (2,3). The presence of BPV-2 DNA has been identified in alimentary fibropapillomas, cutaneous papillomas, and urinary bladder cancer (5,6). BPV types 1 and 2 have also been identified in peripheral blood leukocytes (7,8), and in different body fluids, such as milk, colostrum, and semen, obtained from animals with papillomatosis (5,9,10), and even from apparently healthy cattle (8,11) and horses (2,12). BPV-4 is associated with cutaneous papillomatosis, besides upper-gastrointestinal papillomatosis (6,8,10). Furthermore, BPV-9 and BPV-10 have been identified in squamous epithelial papillomas of the udder, whereas BPV-3 and BPV-8 are responsible for cutaneous papillomas (6).

BPVs can establish latency in cattle (2) and the infection is transmitted to naive animals by milk, semen, and urine or to newborn calves by vertical transmission (13). Polymerase chain reaction (PCR) remains an important tool for diagnostic purposes, particularly in determining asymptomatic carriers within the population, in clinical research, and in field investigations about viral infections (14). The *L1* gene of papillomavirus has been used to detect BPVs by PCR using the degenerated FAP59/64 primer pair, which is the most conserved region (11,15). Nevertheless, the use of degenerated FAP59/64 primers together with type-specific primers is recommended (16,17), since the degenerated FAP59/64 primers have a low sensitivity for detecting new papillomavirus types and are unsuccessful for detecting some BPV types such as BPV-4 and BPV-9 (17).

Bovine leukemia virus (BLV) is an oncogenic deltaretrovirus that belongs to the family *Retroviridae*. BLV

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causes a chronic lymphoproliferative disease characterized by the development of immunosuppression called enzootic bovine leukosis in cattle (18).

Although the identification of BPV types and epidemiological and clinical studies have provided new insights for the prevention and therapeutic procedures of papillomavirus infections (15), little is known about the presence of BPV in Turkey, except *Deltapapillomavirus* (BPV-1 and BPV-2) (19–21). The objective of the present study was to screen for the presence of different BPV types in cattle with papillomatosis as well as in healthy-appearing cattle. This study represents the first large-scale report on the molecular identification and coinfection of BPV types in cattle in Turkey.

2. Materials and methods

2.1. Animals sampled

A total of twenty papilloma-like and three tumor-like lesions from three beef and eight dairy herds in three provinces (Hatay, Adana, and Osmaniye) in Turkey and nine blood samples in EDTA-containing tubes obtained from healthy-appearing cattle from these same herds were examined. A large number of cutaneous samples were taken from two cattle described as "Cattle" in Table 1. The study protocol was approved by the Laboratory Animal Ethics Committee of Mustafa Kemal University, Turkey (No. 2015/8-11).

2.2. Molecular identification

Viral nucleic acid was extracted with a viral nucleic acid extraction kit (Roche, Germany) as described in the manufacturer's instructions. First, PCR was performed, using oligonucleotide FAP59/64 degenerate primers targeting a 478-bp fragment of the *L1* gene of the papillomavirus in the manner described by Ogawa et al. (11) and using primer pairs (forward primer 5'-TTTGTGCATGACCTACGAGCTACA-3' and reverse primer 5'-AAGCGGTCTTCGACTGGAATCT-3'; nucleotides 2464 to 2621) amplifying the *pol* gene of BLV (22). All DNA samples were then identified using BPV type-specific primers (4). Amplified DNA products were visualized under a UV transilluminator and stained with ethidium bromide at 0.5 µg/mL using agarose gel electrophoresis (Figure 1). The DNA extracted from Madin-Darby bovine kidney cells was used as a negative control. The DNA products amplified using the degenerate primers FAP59/64 and type-specific primer sets were used for sequence analysis to confirm some BPV types.

2.3. Histopathological analysis

A total of nine papilloma-like samples were fixed in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Sections were cut to 5 µm in thickness and stained with hematoxylin and eosin (H&E). Microscopic photographs of the specimens were taken by digital imaging system (Olympus DP12-BSW, Tokyo, Japan) assembled in a light microscope (Olympus BX50-F4, Tokyo, Japan).

3. Results

A total of 32 samples, including 23 papillomas and 9 blood samples, were examined for molecular identification of BPV types. BPV DNA was detected in 16 papilloma samples (69.6%) and in all 9 blood samples (100%) by FAP59/64 degenerate primers. Totally 25 out of 32 samples (78.1%) were positive for BPV DNA. Furthermore, a total of 3 cattle with tumor-like lesions (13.0%) were positive for BLV (Table 2).

By BPV type-specific primers, the highest prevalence of BPV-1 was detected in cattle with papilloma-like lesions (86.9%) and in the peripheral blood leukocytes of asymptomatic cattle (100%). The most common types in papilloma-like lesions after BPV-1 were as follows: BPV-4 (47.8%), BPV-2 (30.4%), and BPV-8 (17.4%), whereas BPV types 3, 6, and 9 had lower prevalences than others. None of the animals sampled were positive for BPV types 5, 7, or 10 (Table 2).

Coinfections were present in all farms and overall rates of coinfection were determined in 26 of the examined samples (81.3%). Coinfection with BPV-1/BPV-4/BPV-8 (21.9%) was the most frequently identified using BPV type-specific primers (Table 3).

A total of five fibropapillomas and four papillomas from nine papilloma-like lesions examined were histopathologically diagnosed. In cases of fibropapilloma, fibrous connective tissue had also proliferated with mild mononuclear cell infiltration, hyperemia, and focal hemorrhage. Whirlpools were also observed. In three cases of fibropapilloma, intracytoplasmic inclusions were observed in the stratum spinosum and the basal cells of the epidermis. Mild neutrophil infiltrations with infiltration of lymphocytes were detected in another case of fibropapilloma. Furthermore, cases of papilloma categorized as acanthosis, hyperkeratosis, parakeratosis, and koilocytosis were observed. Hyperemia and hemorrhage were also observed, and one case showed severe neutrophil infiltration, whereas another case revealed mononuclear cell infiltration in the dermis (Figure 2).

4. Discussion

BPV infection is known to be associated with papillomatosis of cattle and horse sarcoids (8,12). A recent study described molecular identification of BPV-1 in cattle with cutaneous papillomatosis in Turkey, but BPV-2 was not detected (20). In the present study, the detection rate of BPV types in papilloma-like samples using BPV type-specific primers was greater than that for FAP59/64 degenerate primers,

Table 1. General information about all samples collected and PCR results.

Herd	Sample	Region	Histopathology	FAP 59/64	BPV types
1 (D)	Sample-1 (T)	Perianal region	-	+	BPV-1, BPV-4, BPV-8, BLV
	Sample-2 (P)	Skin	-	+	BPV-1
	Sample-3 (T)	Vaginal region	-	+	BPV-1, BPV-3, BLV
	Cattle-4 (P)	Skin	-	+	BPV-1, BPV-2, BPV-3, BPV-4
	Sample-5 (T)	Rectal region	-	+	BPV-6, BLV
2 (D)	Sample-6 (P)	Skin	-	+	BPV-1, BPV-8
	Sample-7 (P)	Upper eyelid	-	+	BPV-1, BPV-4, BPV-8
	Sample-8 (P)	Udder	-	+	BPV-1, BPV-4, BPV-8
3 (B)	Sample-9 (P)	Skin	-	+	BPV-1
	Healthy-1	Blood 1	-	+	BPV-1, BPV-4
	Healthy-2	Blood 2	-	+	BPV-1, BPV-4, BPV-8
4 (D)	Cattle-10 (P)	Skin	-	-	BPV-1, BPV-2, BPV-3, BPV-9
	Healthy -3	Blood 3	-	+	BPV-1, BPV-8
5 (B)	Sample-11 (P)	Penis	-	-	BPV-1, BPV-2
	Healthy-4	Blood 4	-	+	BPV-1, BPV-4
	Healthy-5	Blood 5	-	+	BPV-1, BPV-4, BPV-8
	Healthy-6	Blood 6	-	+	BPV-1, BPV-4, BPV-8
6 (D)	Sample-12 (P)	Skin	Fibropapilloma	+	BPV-1
	Healthy-7	Blood 7	-	+	BPV-1, BPV-8
	Healthy-8	Blood 8	-	+	BPV-1, BPV-8
7 (B)	Sample-13 (P)	Skin	Fibropapilloma	+	BPV-1, BPV-2
	Sample-14 (P)	Skin	Fibropapilloma	+	BPV-1, BPV-2
	Healthy-9	Blood 9	-	+	BPV-1, BPV-4, BPV-8
8 (D)	Sample-15 (P)	Skin	Papilloma	-	BPV-4
	Sample-16 (P)	Skin	Fibropapilloma	+	BPV-1, BPV-2, BPV-4
9 (D)	Sample-17 (P)	Udder	-	+	BPV-6, BPV-9
	Sample-18 (P)	Skin	Papilloma	+	BPV-1, BPV-2, BPV-4
10 (D)	Sample-19 (P)	Udder	-	+	BPV-1, BPV-4
	Sample-20 (P)	Udder	-	-	BPV-1, BPV-4
11 (D)	Sample-21(P)	Skin	Fibropapilloma	-	BPV-1, BPV-4
	Sample-22 (P)	Skin	Fibropapilloma	-	BPV-1, BPV-4
	Sample-23 (P)	Skin	Fibropapilloma	-	BPV-1

D: Dairy (♀); B: Beef (♂).
T: Tumor-like; P: Papilloma-like.

like the results described by Silva et al. (16). Furthermore, 7 BPV types, including genera *Deltapapillomavirus* (BPVs 1 and 2), *Xipapillomavirus* (BPVs 3, 4, 6, and 9), and *Epsilonpapillomavirus* (BPV-8), were identified. BPV-1 was also the most frequent genotype both in cattle with papilloma-like lesions and in the peripheral blood leukocytes of asymptomatic cattle, followed by BPV-4, BPV-8, and BPV-2. BPV-8 and BPV-4 were found in peripheral blood leukocytes; to the best of our knowledge, this is the first description in peripheral blood leukocytes of asymptomatic cattle for BPV-8. Interestingly, BPV-6 was detected in a tumor-like sample obtained from the rectal region, which was coinfected with BLV, described as an oncogenic retrovirus (18), although BPV-6 is known to be

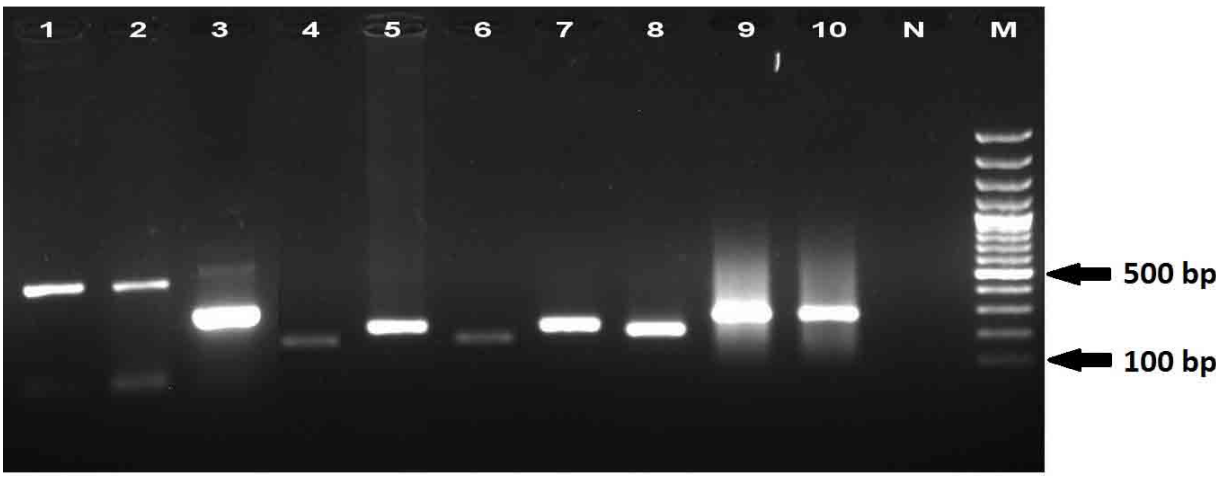

Figure 1. The PCR results with FAP59/64 degenerated and BPV type-specific primers.
Line M: 100-bp molecular weight marker (M) (Roche, Germany), Line N: negative control, Lines 1 and 2: DNA amplification products with FAP59/64 primers (478 bp), Line 3: BPV-1 (301 bp), Line 4: BPV-2 (164 bp), Line 5: BPV-3 (216 bp), Line 6: BPV-4 (170 bp), Line 7: BPV-6 (216 bp), Line 8: BPV-8 (196 bp), Lines 9 and 10: BPV-9 (264 bp).

Table 2. Individual distribution of BPV types in the samples collected from cattle.

BPV types	Papilloma (%) n: 23	Blood (%) n: 9	Total (%) n: 32
BPV-1	20 (86.9)	9 (100)	29 (90.6)
BPV-2	7 (30.4)	-	7 (21.9)
BPV-3	2 (8.7)	-	2 (6.3)
BPV-4	11 (47.8)	6 (66.7)	17 (53.1)
BPV-6	2 (8.7)	-	2 (6.3)
BPV-8	4 (17.4)	7 (77.8)	11 (34.4)
BPV-9	2 (8.7)	-	2 (6.3)
BLV	3 (13.0)	-	3 (9.4)

Table 3. Coinfection groups in all samples collected from cattle.

Coinfection	Papilloma (%) n: 23	Blood (%) n: 9	Total (%) n: 32
BPVs 1 and 2	3 (13.0)	-	3 (9.4)
BPVs 1 and 3	1 (4.3)	-	1 (3.1)
BPVs 1 and 4	4 (17.4)	2 (22.2)	6 (18.7)
BPVs 1 and 8	1 (4.3)	3 (3.3)	4 (12.5)
BPVs 6 and 9	1 (4.3)	-	1 (3.1)
BPVs 1, 4, and 8	3 (13.0)	4 (44.4)	7 (21.9)
BPVs 1, 2, and 4	2 (8.6)	-	2 (6.2)
BPVs 1, 2, 3, and 4	1 (4.3)	-	1 (3.1)
BPVs 1, 2, 3, and 9	1 (4.3)	-	1 (3.1)

the principal cause of the development of udder papilloma (1). Moreover, different BPV types were also identified in the blood of healthy cattle and cattle with papilloma-like samples housed together in the same herd. However, some studies have indicated that papillomaviruses can be disseminated to nonepithelial tissues through the

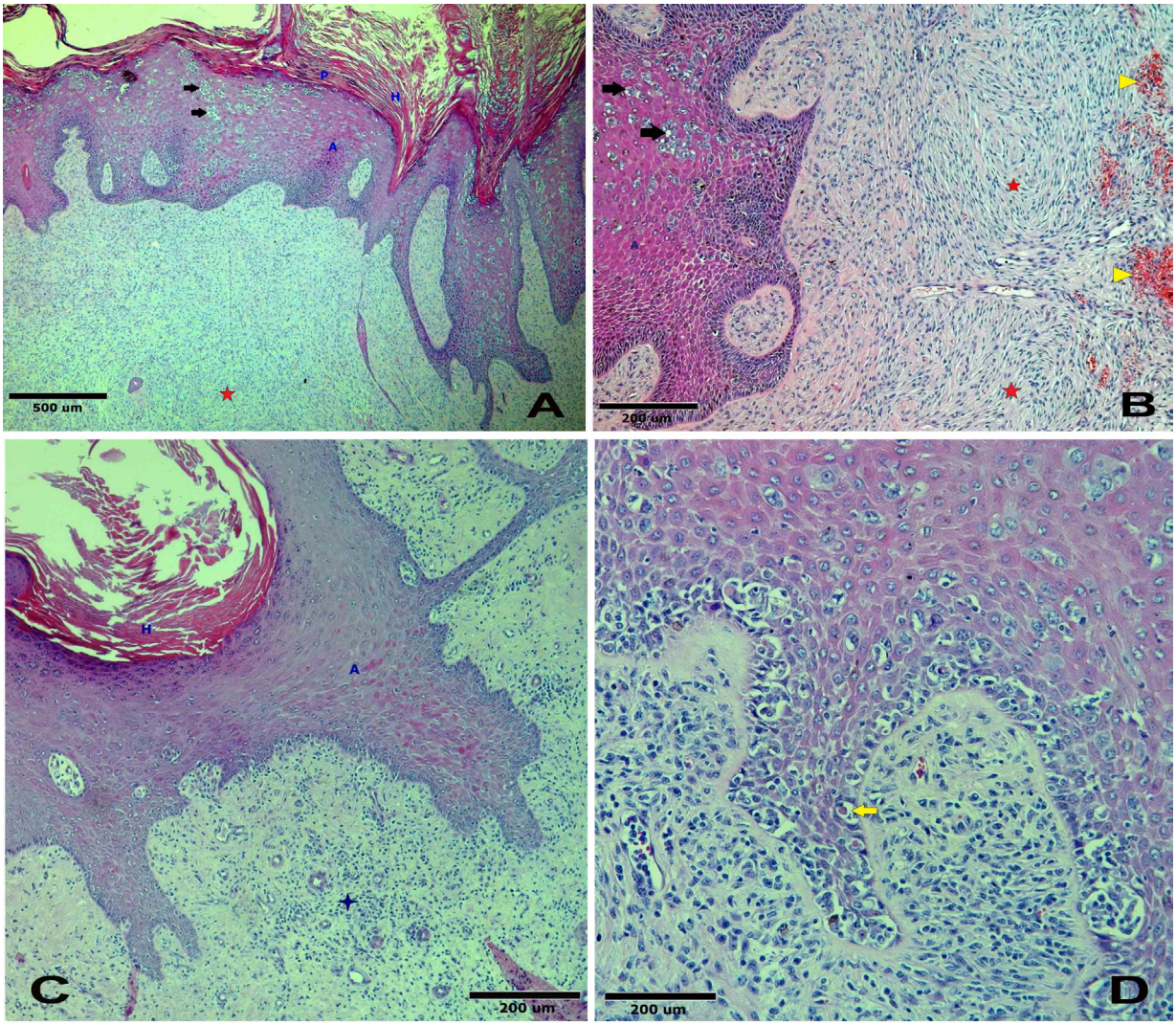

Figure 2. Fibropapilloma (A, B, D): acanthosis (A), hyperkeratosis (H), parakeratosis (P), vacuolar degeneration (arrows), and intracytoplasmic inclusions in basal cells (arrows) in epidermis, whirlpools in hyperplasia of the fibrous connective tissue (stars), and hemorrhage (arrow heads) in dermis. Papilloma (C): acanthosis (A), hyperkeratosis (H), koilocytes including keratohyalin granules in epidermis, mononuclear infiltration (star) in dermis.

bloodstream as a result of infection through semen, milk, urine, or reproductive tissues (7,10,13). It has been suggested that different papillomaviruses may circulate constantly in both symptomatic and asymptomatic cattle housed in the same herd, and these asymptomatic animals could also play a major epidemiological role in the transmission and persistence of the virus.

Our results suggest that coinfection of BPVs is common in both symptomatic cattle and healthy-appearing cattle. This result might be linked to the persistence of the virus as described by Grindatto et al. (17). Coinfections with two, three, and four BPV genotypes were determined in 46.9%, 28.1%, and 6.3% of cattle, respectively, whereas single infection was detected in 18.8%. Nevertheless, immunological failure might also have played a predisposing role in the dissemination of BPVs (6). In the present study, BLV was identified in three cattle with tumor-like lesions sampled from the same herd (13.0%), which causes immune suppression (18). None of other BPV-identified samples were negative for BLV, but we have not examined other immunological or physiological adverse factors in these animals.

In this study, five fibropapillomas and four papillomas from nine cases examined were diagnosed, and in all cases varying degrees of epidermal proliferation and irregular papillary extension due to expansion of stratum spinosum cells were seen. In the epidermis, mild to severe acanthosis, hyperkeratosis, prominent parakeratosis, mild

to moderate basophilic cytoplasmic swelling, vacuolar degeneration of keratinocytes, and many koilocytes (including keratohyalin granules) were present. Moreover, similar histopathologic features in BPV infection have been previously reported in cattle from Brazil (15,23,24) and from Italy (17,25), and in water buffaloes and cattle from Turkey (19–21) and from India (26).

In conclusion, our study provides general epidemiological information about BPV infection in Turkey, which will be highly useful for guiding further large-scale epidemiological studies. However, further studies are needed to determine the risk factors associated with BPV infection in cattle populations in Turkey.

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