Investigation of the Presence of Bovine Papillomavirus in Blood Leukocytes of Cattle in West Mediterranean of Turkey

ABSTRACT

One hundred Holstein cattle, varying in age from one to ten years, who had warts on different parts of its bodies and had previously had Bovine papillomavirus (BPV) typing had blood samples taken from them from various managements in Burdur-Center and its surroundings. DNA was extracted from leukocytes using the DNeasy Blood & Tissue Kit (Qiagen, Germany). RT-PCR test was performed using consensus and type-specific primers for the detection of BPV types. As a result of the study, RT-PCR test and consensus primers were not detected in blood leukocyte samples. Through the use of type-specific primers in an RT-PCR assay, the viral genome components of BPV-10 (56), BPV-11 (1), BPV-10+BPV-11 (2), BPV-10+BPV-12, and BPV-10+BPV-14 (2) were detected in these samples. BPV-12 was detected for the first time in blood leucocytes.In blood samples taken from animals classified according to the anatomical regions where the warts are located the highest BPV positivity (22,2%) was detected in animals with warts on the teats. Also, the most BPV-10 type was determined in the blood samples of animals with wart lesions on the teat and lobes. When the distribution of BPV PCR positive samples according to age groups was made, BPV PCR positive was found mostly in the between 1 and 47 month age group (71,4%). It was determined that the prevalence of BPV was high in the between 1 and 47 age group, while the rates of 48-83 months and later decreased with increasing age.In this investigation, it was found that BPV type 10 had been prevalent in blood leukocyte samples from calves with warts in various areas of the body and with variable macroscopic appearance.

Keywords: Bovine papillomavirus, Blood, Leucocyte, PCR, Cattle, Wart.

1. INTRODUCTION

Livestock farming is among the most important industries in the world and in Turkey. There are 1.5 billion cattle in the world and approximately 18 million in Turkey. In the production of bovine products in Turkey, 90% of red meat and 91% of milk are obtained from cattle [1,2].

One of the important health problems in cattle farms is warts that develop due to BPV infection.Papillomatosis cases caused by papillomavirus (PV) have an important place in cattle[3]. Warts seen on the skin and mucosa easily spread to other body parts and can progress to tumoral structures if left untreated [4].There are many direct and indirect ways for the disease agent to be transmitted to cattle [5].Skin and blood are the most frequently used as entrance ways [6].In cattle, warts spread to areas such as the eyelids, ears, between the nails, mouth, tongue, lips, gums, vagina, udder and penis, and can cause functional disorders.Appearance and deformities in animals with warts affect animal sales and, in this context, lead to economic losses [7,8].

In this research, various areas of the body [eye, ear, nose, between the nails, mouth, lip, tongue, gum, vagina, penis, teat, teat lobe, head, body, eyelid] were collected from various enterprises owned by the public in Burdur-Center and its districts. Blood samples were collected from 100 cattle (Holstein breed, aged between 1 and 10 years) with warts of different appearance. The presence of BPV in the obtained blood leukocytes was investigated by polymerase chain reaction (PCR) test using consensus and type-specific primers.The results obtained were determined and interpreted according to anatomical wart formation areas in the animal, virus types obtained and age.

2.MATERIAL AND METHODS

2.1. Sampled Animals

Various areas of the body [eye, ear, nose, between the nails, mouth, lip, tongue, gum, vagina, penis, teat, teat lobe, head, body, eyelid] were collected from various enterprises owned by the public in Burdur-Center and its districts. Blood samples were collected from 100 cattle (Holstein breed, aged between 1 and 10 years) with warts of different appearance (Table 1).

Table 1. Anatomical distributions of wart lesions in animals subjected to blood sampling

Anatomical distributions	Papilloma	
	Numbers	**%**
Eye (Eyelid)	2	2
Ear	2	2
Nose	5	5
Mouth	5	5
Vagina	6	6
Penis	8	8
Teat	20	20
Mammary lobes	12	12
Head	8	8
Body	12	12
Tail	10	10
Leg	7	7
Feet (Interdigital Region)	3	3
Total	**100**	**100**

2.2. Blood Sampling

Blood samples of the animals to be sampled were taken from the jugular vein, 10 ml, and transferred to K_3EDTA tubes. After the blood samples were brought to the laboratory under cold chain, they were centrifuged at 2000 rpm for 20 minutes. At the end of the centrifugation period, the erythrocytes at the bottom and the leukocyte layer called Buffy Coat, which formed a white cloud in the middle of the plasma layer at the top, were removed with a pasteur pipette. The obtained leukocytes were washed 3 times with PBS. Leukocytes were stored in a -80 ˚C deep freezer until extraction.

2.3. DNA Extraction

DNA was extracted from leukocytes using the DNeasy Blood & Tissue Kit (Qiagen, Germany). The application was performed according to the protocol (Nucleated blood step) reported by the manufacturer. The resulting DNA extract was stored in a -80 ˚C deep freezer until PCR application.

2.4. PCR with the use of consensus primers

Consensus primers FAP59/64 (Fw 5'-TAACWGTIGGICAYCCWTATT-3'; Rev 5'CCWATATCWVHCCATITCICCATC-3') and MY11/09 (Fw 5'-GCM CCM ARR GGA WAC TGA TC-3'; Rev 5'-CGT CCM ARR GGA WAC TGA TC-3') are used for the detection of a wide range of BPV types. These primers were prepared by combining them in the procedure given below. In this way, the L1 Open Reading Frame (ORF) protected fragment was amplified [9].

2.4.1. Procedure

1. PCR mix was prepared by adding Taq DNA polymerase 25 units/ml, 200 µM dATP, 200 µM dGTP, 200 µM dCTP, 200 µM dTTP, 1.5 mM MgCl2 and 0.25µM each oligonucleotide primer (FAP59/64 and MY11/09).

2. This mixture was homogenized with a pipette and 23 µl was transferred to each PCR tube.

3. 2 µl of the DNA sample at a concentration of 50 ng/µl was added and the mixture was homogenized with a pipette.

4. 2 µl of ultrapure water was added to a PCR tube and used as a nontemplate control (NTC).

5. The samples were placed in the thermocycler device and the DNA fragments were amplified. The PCR Thermal Profile of the Consensus Primers used was made in the table below (Table 2).

6. Amplification products were demonstrated using 1.5% Tris acetate buffer (TAE) agarose gel electrophoresis and ethidium bromide dye [9].

Table 2. PCR thermal profile of consensus primers

Primer	Denaturation	Annealing	Extension	Cycles
FAP59/64	1 min. 94 ˚C	1 min. 50 ˚C	1 min. 72 ˚C	45
MY11/09	30 sec. 95 ˚C	30 sec. 48 ˚C	1 sec. 72 ˚C	35

2.5. PCR using type-specific primers

BPV type specific primers were prepared by combining them in the procedure given below:PCR mix was prepared by adding Taq DNA polymerase 25 units/ml, 200 µM dATP, 200 µM dGTP, 200 µM dCTP, 200 µM dTTP, 1.5 mM MgCl2 and 0.25µM of each oligonucleotide primer (Table 3).

Table 3. 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 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	
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 gene
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

This mixture was homogenized with a pipette and 23 µl was transferred to each PCR tube. 2 µl of the DNA sample at a concentration of 50 ng/µl was added and the mixture was homogenized with a pipette. 2 µl of ultrapure water was added to a PCR tube and used as a nontemplate control (NTC). The samples were placed in the thermocycler device and the DNA fragments were amplified. Accordingly, PCR Thermal Profiles of various BPV primers used (except BPV-7) were performed (Table 4). Amplification products were demonstrated by 2% TAE agarose gel electrophoresis and the use of ethidium bromide dye [9].

Table 4. PCR thermal profiles of BPV primers

Primers	Denaturation	Annealing	Extension	Cycles
BPV-1	40 sec. 94 ˚C	40 sec. 68 ˚C	1 min. 72 ˚C	35
BPV-2	40 sec. 94 ˚C	40 sec. 55 ˚C	1 min. 72 ˚C	35
BPV-3	40 sec. 94 ˚C	40 sec. 60 ˚C	1 min. 72 ˚C	35
BPV-4	40 sec. 94 ˚C	40 sec. 60 ˚C	1 min. 72 ˚C	35
BPV-5	40 sec. 94 ˚C	40 sec. 60 ˚C	1 min. 72 ˚C	35
BPV-6	40 sec. 94 ˚C	40 sec. 65 ˚C	1 min. 72 ˚C	35
BPV-8	40 sec. 94 ˚C	40 sec. 60 ˚C	1 min. 72 ˚C	35
BPV-9	40 sec. 94 ˚C	40 sec. 60 ˚C	1 min. 72 ˚C	35
BPV-10	40 sec. 94 ˚C	40 sec. 60 ˚C	1 min. 72 ˚C	35
BPV-11	40 sec. 94 ˚C	40 sec. 54 ˚C	1 min. 72 ˚C	35
BPV-12	3 mins. 95 ˚C pre-denaturation (1 time) 30 sec. 95 ˚C	30 sec. 60 ˚C	5 mins. 72 ˚C	35
BPV-13	5 mins. 94 ˚C pre-denaturation (1 time) 45 sec. 95 ˚C	45 sec. 60 ˚C	5 mins. 72 ˚C	35
BPV-14				

2.6. Statistical analysis

In this study BPV (Type Specific Primers) PCR test results, PCR positivity distributions where single and mixed types occur together (co-infection), BPV positivity and type distributions in blood samples taken from animals where warts were classified according to their anatomical regions and descriptive statistics were performed for the distribution of BPV PCR positive samples according to age groups and the numerical values obtained were given as percentages. SPSS 15.0 for Windows® (IBM Software) analysis program was used for statistical analysis.

3.RESULTS

3.1. BPV PCR Test Results

Consensus primers FAP59/64 and MY11/09 were used to detect a wide range of BPV types.No positivity was detected in the PCR test performed with FAP59/64 and MY11/09 consensus primers in 100 blood leukocyte samples.

BPV type specific primers BPV-1 (L1), 2 (L1), 3 (L1), 4 (E7), 5 (L1), 6 (L1), 8 (L1), 9 (L1), 10 (L1).), 11 (L1), 12 (L1), 13 (L1), 13 (E5) and 14 (L1) were used.Accordingly, in general, BPV positivity was detected in 63 out of 100 samples (Table 5). The most uniform positivity distribution in the samples was determined as 56 (88.8%) BPV-10 (Table 6). Additionally, 1 (1.6%) BPV-11 positivity was found in a single type of samples (Table 6). In other samples, two types of positivity distribution was determined as; 2 (3.2%) BPV-10+BPV-11, 2 (3.2%) BPV-10+BPV-12 (Table 6) and 2 (3.2%) BPV-10+BPV-14 (Table 6).

Table 5. BPV (Type Specific Primers) PCR test results

Blood Leukocyte	BPV (+; %)	BPV (-; %)	Total
BPV PCR	63 (63%)	37 (37%)	100

Table 6. PCR positivity distributions where single and mixed types occur co-infection

Single and Mixed Types	BPV (+; %)
BPV-10	56 (88,8%)
BPV-11	1 (1,6%)
BPV-10, 11	2 (3,2%)
BPV-10, 12	2 (3,2%)
BPV-10, 14	2 (3,2%)
Total	63 (100%)

One hundred Holstein dairy cattleraised for milk were used in the research. In the blood samples taken from animals where warts were classified according to their anatomical regions, the highest BPV positivity (22.2%) was detected in animals with warts on the teat (Table 7). In addition, it was determined that the most BPV type in the blood samples taken from the animals was 10, and these animals had wart lesions on the teat and lobes (Table 7).

Table 7. BPV positivity and type distributions in blood samples taken from animals where warts were classified according to their anatomical regions.

Anatomical Placement	BPV (+) Samples		BPV Types Samples			
	N	%	10	11	12	14
Eye (Eyelid)	1	1,6	-	1	-	-
Ear	1	1,6	1	-	-	-
Nose	2	3,1	2	-	-	-
Mouth	2	3,1	2	-	-	-
Vagina	5	7,9	5	-	-	-
Penis	6	9,5	6	-	-	-
Teat	14	22,2	10	2*	2**	-
Mammary lobes	10	16	10	-	-	-
Head	4	6,3	4	-	-	-
Body	10	16	8	-	-	2***
Tail	3	4,8	3	-	-	-
Leg	2	3,1	2	-	-	-
Feet	3	4,8	3	-	-	-
Total	63	100	56	3	2	2

BPV-10+BPV-11; ** BPV-10+BPV-12; * BPV-10+BPV-14*

In the study, BPV PCR positive was found mostly in the 1-47 month age group (71.4%). Distribution ofpositive and negative samples according to age groups is shown in Table 8.

Table 8. Distribution of BPV PCR positive samples by age groups.

Age groups	BPV (+; %)	BPV (-; %)
1-47 months	45 (71,4%)	14 (37,8%)
48-83 months	13 (20,6%)	16 (43,2%)
84 months and above	5 (8%)	7 (19%)
TOTAL	63 (100%)	37 (100%)

4.DISCUSSION

Primers FAP59/64 and MY09/11 are used for PV identification in humans, cattle and other animals [10]. These primers were designed originating from conserved regions of the HPV L1 gene [11]. Özmen and Kale [8] determined FAP59/64 positivity in teat warts, but found all samples negative with MY11/09 consensus primer. Melo et al. [12] sequenced DNA of BPV-1, 2 and 4 in blood samples and detected virus co-infection in all blood samples.However, they observed that BPV FAP59/64 primers were not detected in blood samples. Silva et al. [13] reported that although FAP59/64 degenerate primers were ideal for the basic viral type and new PV, their sensitivity was lower than other specific primers and this was due to the fact that they were prepared based on the HPV target. In this study, no positivity was detected in the PCR test performed with FAP59/64 and MY11/09 consensus primers in 100 blood leukocyte samples.

In our study, BPV positivity was detected in 63 of 100 samples using the PCR test and BPV type-specific primers between BPV-1 to BPV-14 (except BPV-7). The most uniform positivity distribution in the samples was determined as 56 (88.8%) BPV-10. Among the samples, 1 (1.6%) BPV-11 in one type, 2 (3.2%) BPV-10+BPV-11 in two types, 2 (3.2%) BPV-10+BPV-12, 2 (3.2%) BPV-10+BPV-14 were found. There are many studies that differ from the BPV type-specific results we obtained in blood samples in our study [14, 15].

The existence of BPV-10 [8,16], BPV-11 [17] and BPV-14 [18] types, which we obtained specific for BPV type in blood samples in our study, has also been demonstrated by several researchers. However, in our literature review, we could not find any research on the detection of BPV-12 in blood samples. BPV-10, 11 and 12 are included in Xipapillomavirus [19,20]. Except for the BPV types (BPV-10, 11, 12 and 14) that we identified in blood samples in the study, no other types were identified. We estimate that this may be due to the low incidence of BPV types in the sampled area, as stated by Diniz et al. [15] and Santos et al. [21], or to the fact that these virus types are present in the blood in lower amounts, resulting in a transient infection.

As a result of transfusion of BPV-containing blood to BPV-free cows, the presence of BPV DNA was determined in the peripheral blood mononuclear cells of these cows. In addition, the presence of the virus has been detected in the offspring of these animals. According to these results, it was concluded that the virus can be transmitted *in utero* way [22]. Freitas et al. [6] stated that BPV types spread to all parts of the body through blood, and thus the circulating BPV types cause new skin lesions. They also expressed the association that BPV co-infected blood causes horizontal [22] and vertical [23] transmission, as reported by other researchers. It was concluded that there is a relationship between the BPV DNA detected in peripheral blood lymphocytes and the host immune system and the virus. They emphasized that lymphocytes are not only derived from latent sites of infection but may also be a potential vector for BPV distribution among animals [24]. While it is estimated that the presence of the virus in the blood may cause infection in asymptomatic animals, it has been reported that symptoms develop as a result of tissue injuries in infected animals [25].

In our study, in the blood samples taken from animals whose warts were classified according to their anatomical regions, the highest BPV positivity was observed in animals with warts on the teat, mammary lobes and body. However, BPV-10 was detected mostly in the blood samples of animals with warts on their teats and lobes. Hamad et al. [26] reported that BPV-9 and BPV-10 types were associated with epithelial squamous papilloma in the breast lobes. Hamad et al. [27] determined the presence and prevalence of BPV in warts located in different parts of the body, with the highest level in the bovine udder and neck. Similar results were reported by De Villiers et al. [28].

BPV affects cattle of all ages. However, cattle under 3 years of age are more affected by the disease [30]. It has been stated that breast warts are most common between the ages of 3-8 [31]. Hamad et al. [27] found a high prevalence of BPV in female cattle. They attributed this to pregnancy, lactation and keeping the animals by breeding for a long time in females, and keeping the animals for less than 2 years in males and sending the animals to slaughter in a short time due to raising them for meat production.It has been reported that alkaline skin pH at young ages, incomplete development of the immune system, disappearance of maternal antibodies from the mother, development of immune response against other pathogens, sensitivity to parasitic infestations, and stress factors all contribute to the prevalence of BPV infection in young people [32]. In our study, BPV PCR positive blood samples were found mostly in the 1-47 month age group (71.4%). In addition, the study found that while the prevalence of BPV was high in the 1-47 month age group, the rates in the 48-83 month and later age group decreased with advancing age.

5.CONCLUSION The common presence of BPV-10 and, to a lesser extent, BPV-11, BPV-12 and BPV-14 types was detected in the blood leukocytes of cattle with wart lesions in Burdur Center and its districts. BPV-12 was detected in blood leukocytes for the first time. We recommend that these types be sequenced and phylogenetically analyzed in the future. We also estimate that other types that cannot be detected in blood leukocytes are present in lower levels in the bloodstream. In the study, we determined that the highest positivity in blood leukocytes was in animals with warts on the teat and lobes. We attribute the main reasons for this to the following: animals are exposed to a large number of treatment applications due to dairy cattle breeding, deformation and traumatic injuries in the udder as a result of using milking machines to milk twice a day during the lactation period, calves sucking their mothers' teats after birth, failure to pay attention to hygiene rules before and after milking, and portable milking machines are usually carried out in the living area. However, a number of factors may indirectly contribute to the occurrence of the disease: frequent animal purchases from outside to farms, intensive artificial insemination practices, mating with bulls, due to the geographical location of the region (temperate climate, lakes region), fly population density, body injuries caused by the environment, failure to treat the injured area, and ignoring treatments for warts that occur. In the next stage of this study, we recommend that detailed studies be carried out to evaluate the disorders occurring at the chromosomal level in blood samples taken from BPV-infected animals together with field experts, to reveal the causes and to eliminate these causes.

ETHICAL APPROVAL

Samples from animals were collected in accordance with the bioethical and standard procedures of the "Bioethics Committee of the MAKÜ HADYEK (15.09.2021/Protocol No:807)"

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