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Orf Virus (ORFV) Infection in the Mouth of Lambs and Kids Detected by Molecular Investigation

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ABSTRACT

***Background*:** Contagious Ecthyma infections caused by *Parapoxvirus ovis* are an important viral disease of sheep and goats. It is an infection with high mortality that especially affects young animals. The majority of Orf virus (ORFV) infections are diagnosed using molecular diagnostic techniques (multiplex, conventional, real-time PCR). Scab samples were collected from lesions developed due to ORFV in the mouths of 50 lambs and 50 kids that were not vaccinated against ecthyma disease from public pens in Burdur-Center and surrounding areas. The main objective of the study was to investigate the ORFV genome in the samples using the conventional PCR method with 25 different types of primers.

***Materials, Methods & Results*:** Scab samples were collected from lesions defined because of ecthyma disease in the mouth (lips, gums) of 50 lambs and 50 kids aged ≤ 1 month to 3 months in various public pens. In the study, high viral genome positivity was determined in local breed lambs [Sakiz sheep (40%) and kids - Kil goat (36%)]. ORFV genome presence was detected more positivity in female lambs (60%) and kids (46%) than in males. ORFV genome presence was detected more positivity in lambs (36%) and kids (40%) aged ≤ 1 month, than in those in the 2- and 3-month age group. In this study, because of PCR applications, ORFV was predominantly detected in the Burdur region; GIF/IL2, PPP1-PPP4, Orf1-Orf2, VIR1 VIR2, vIL-10-3 vIL-10-4, B2L and Alpha tubulin primers was detected. No positivity was obtained in PCR analysis using PPV JGR, ORFV 011-B and ORFV 109 primers. In our study, it was found that the groups with the most binary ORFV primers were found at the highest rate in the tissues.

***Discussion*:** Contagious Ecthyma infection caused by *Parapoxvirus ovis* is an important viral disease of sheep and goats. It is an infection with high mortality that especially affects young animals. The infection primarily affects animals under 1 year of age, and it also affects animals 3 to 4 months after birth. Therefore, it is a disease that causes both economic and productivity loss. Due to factors like weight loss and weakened immunity brought on by limited amounts of breast milk and feed intake because the lesions are in the mouth, ecthyma has a more severe effect on lambs and kids than it does on adults. The susceptibility of animals to secondary infections may increase. Young animals die at a higher rate as a result. The mortality of the majority of the offspring in that herd at that time and the development of productivity losses are indicators of the spread of ORFV in small cattle farms over time. Many classical diagnosis methods are used in the disease. Molecular diagnostic methods are mainly used in ORFV infections because they are sensitive, rapid and low-cost. One of these is the PCR application, which is used worldwide and in Turkey and yields findings more quickly than other techniques. In the research, the presence of viral genome was investigated by PCR using 25 different types of primers in the oral lesions of lambs and kids. In this study, the presence of ORFV in lesions occurring in the mouth area of lambs and kids was investigated molecularly. In the current study, ORFV was detected in most of the 7 primers, and the groups with the most binary ORFV primers were found at the highest rates in the tissues. As a result, the presence of ORFV was detected molecularly in the lesions occurring in the mouth area of lambs and kids of different breeds.

Keywords: ecthyma, *Parapoxvirus ovis*, Orf virus, PCR, molecular diagnostic, kid, lamb.

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INTRODUCTION

The disease may be called contagious pustular dermatitis, infectious pustular dermatitis, scabby mouth, contagious pustular stomatitis, contagious ecthyma or simply ecthyma in sheep and goats [16]. ORFV enters the body through damaged skin and multiplies in epidermal cells. Apart from this, it has been reported that clinically healthy sheep can transmit the disease to other sheep following the dipping process [24].

The disease usually affects animals younger than 6 months and newborn animals. It is not possible for newborn lambs and kids with lesions in the mouth area to receive colostrum [5]. Colostrum is a very important nutritional source for the immune system of newborn lambs and kids [5]. The mortality rate of baby animals that are deprived of colostrum or have insufficient breastfeeding increases significantly because the immune system is not sufficiently developed and the antibody titer due to the virus is not formed [5]. The clinical symptom worsens in young animals due to oral lesions and secondary infections that develop accordingly. The most important way to achieve economic gain in sheep and goat herds is to purchase healthy offspring and continue to increase the size of the herd. Ecthyma directly affects breeders economically as it increases mortality in young animals [4,5].

The clinical appearance of the lesions is important for the diagnosis of ORFV. Serological, virological, histopathological, electron microscopic and molecular diagnostic methods of the agent are used to diagnose ORFV [23,25].

The aim of the study was to detect ORFV using different primers in lambs and kids. Also, it was to determine which primers were most prevalent.

MATERIALS AND METHODS

Samples

Scab samples were taken from the lesions developed because of ORFV in the mouths (lips, gums) of 50 lambs and 50 kids that had not been vaccinated against ecthyma disease, from various public-owned pens in Burdur-Center and its districts. The collected samples were stored in a -80°C for molecular diagnosis. Age (Table 1), gender (Table 2) and breed (Table 3) characteristics of the sampled animals are given in tables.

Table 1. The distributions of number (N) of age (month) in the lambs and kids.

Lambs	N
≤ 1 month	18
2 months	15
3 months	17
Total	50
Kids	N
≤ 1 month	20
2 months	17
3 months	13
Total	50

Table 2. The gender (♀/♂) distribution in the lambs and kids.

Lambs	N
Female	30
Male	20
Total	50
Kids	N
Female	25
Male	25
Total	50

Table 3. The distributions of number (N) of breed in the lambs and kids.

Lambs	N
Sakiz sheep	20
Anatolian Merinos	15
Kivircik sheep	15
Total	50
Kids	N
Kil goat	20
Damascus goat	15
Maltiz goat	15
Total	50

DNA Extraction

The collected scab samples (20 mg) were cut into small pieces with a scalpel and placed in sterile eppendorf tubes. DNeasy Blood & Tissue Kit[1] was used for DNA extraction. The application was made according to the protocol stated by the manufacturer. The obtained DNA extracts were stored at -80°C until PCR application.

PCR

In this study, ecthyma samples collected from lambs and kids in Burdur Center and public farms were typed by using 25 different types of primers (Table 8) from Metabion[2] in PCR applications.

Type primer preparation and sample processing procedures

Primers were diluted according to the protocol specified by the company. It was prepared for PCR mixes by performing molar modifications using the procedures specified (Table 4). For each sample, a PCR mix of 50 µL (5 µL Taq Buffer, 5 µL dNTP Mix, 3 µL MgCl2, 0.6 µL Taq DNA Polymerase, 1 µL Forward, 1 µL Reverse, 30 µL DNase/RNase Free Water, 5 µL Template) was made according to the Thermo Scientific protocol. PCR mixes were processed in a thermocycler[3] in accordance with the information specified in the literatures. In the samples we prepared, DNA fragments were amplified in line with the values specified in the literatures. The products amplified in the thermal cycler device[3] were carried out in 1.5% TAE agarose gel electrophoresis with safe view dye[4] and imaged with Bio Rad GelDoc Go Imaging System[5].

Statistical analysis

Statistical analysis of the data was performed with IBM SPSS Statistics 27 analysis software. Numerical data were shown as percentages. Differences in nominal data between categorical groups were analyzed by the Chi-Square test. The statistical significance level was taken as $P < 0.05$.

Table 4. ORFV type specific primers, amounts and sample processing procedures.

Procedure	Primer	Primer pMol	Primer Lettering	Reference
Procedure I	GIF/IL-2	20 pMol	A	[21]
Procedure II	PPP1-PPP4	10 pMol	B	[15]
	PPP3-PPP4	10 pMol	C	
	ATI gene primer	10 pMol	D	
Procedure III	Orf 1-Orf 2	10 pMol	E	[1]
Procedure IV	ORFV-B2L	20 pMol	F	[13]
	ORFV-A32	20 pMol	G	
Procedure V	vIL-10-3 vIL-10-4	25 pMol	H	[3]
	OVA32 LF1 OVA32 LR1	25 pMol	I	
	VR1 VR2	25 pMol	İ	
Procedure VI	PPV JGR	10 pMol	J	[6]
Procedure VII	ORFV 011-A	10 pMol	K	[7]
	ORFV 059-A	10 pMol	L	
	ORFV 011-B	10 pMol	M	
	ORFV 059-B	10 pMol	N	
	ORFV 109	10 pMol	O	
	ORFV 110	10 pMol	P	
	ORFV 127	10 pMol	R	
Procedure VIII	045 gene	10 pMol	S	[9]
Procedure IX	B2L gene primer	10 pMol	T	[14]
	F1L gene primer	10 pMol	U	
Procedure X	Alpha-tubulin	25 pMol	Ü	[17]
Procedure XI	From B2L gene primers	10 pMol	V	[28]
Procedure XII	ORFV 011-C (B2L)	10 pMol	Y	[31]
	ORFV 059-C (F1L)	10 pMol	Z	

RESULTS

Based on the results of PCR tests, the distribution of Orf virus in lambs and kids sampled for the study was shown according to breed (Table 5), gender (Table 6), and age (Table 7). No positive results were obtained in 3 of the 25 primers we used. PCR results was determined according to primers (Table 8). As a result of testing all primers in 100 samples, the positivity rate was found to be 94%. Single or multiple primer distribution and percentage ratios was demonstrated in Figure 1.

A total of 60% of the lambs were female and 1.98 ± 0.8 months old. Among the primers used in ORFV detection, significant results were found in the GIF/IL-2 and B2L gene primers ($P = 0.009$; $P < 0.05$). These are Sakiz sheep, which were positive at the GIF/IL-2 gene (48.6%) and B2L gene primer (53.8%), and Anatolian Merinos, which were negative at the GIF/IL-2 (60%) and B2L gene primer (45.8%). At the same time, the GIF/IL-2 gene was positive in 1-month-old lambs (42.9%), and the B2L gene was positive in 2-month-old lambs (50%). In female lambs, PPP1-PPP4 primer positivity (72.4%) [$P = 0.035$; $P < 0.05$] and negativity for the ORFV-A32 gene primer (71%) were significant [$P = 0.045$; $P < 0.05$].

Goats were on average 1.86 ± 0.8 months old. There was no significant difference in the PCR procedures for ORFV detection according to sex or breed ($P > 0.05$). However, the results of Orf 1-Orf 2 (44.4%) [$P = 0.028$; $P < 0.05$], vIL-10-3 vIL-10-4 (66.7%) [$P = 0.009$; $P < 0.05$], and B2L gene primer (70.0%) [$P = 0.016$; $P < 0.05$] were positive in 1-month-old goats. There was no significant difference in the results of the primers used to determine ORFV by PCR in goats by gender or breed ($P > 0.05$).

Table 5. The distribution of PCR test results according to breeds in lambs and kids.

	Positive		Negative	
Lambs	N	%	N	%
Sakiz sheep	20	40 %	-	0 %
Anatolian Merinos	14	28 %	1	2 %
Kivircik sheep	15	30 %	-	0 %
Total	50	98 %	1	2 %
Kids	N	%	N	%
Kil goat	18	36 %	2	4 %
Damascus goat	13	26 %	2	4 %
Maltiz goat	14	28 %	1	2 %
Total	50	90 %	5	10 %

Table 6. The distribution of PCR test results according to gender (♀/♂) in lambs and kids.

	Positive		Negative	
Lambs	N	%	N	%
Female	30	60 %	0	0 %
Male	19	38 %	1	2 %
Total	49	98 %	1	2 %
Kids	N	%	N	%
Female	23	46 %	2	4 %
Male	22	44 %	3	6 %
Total	45	90 %	5	10 %

Table 7. The distribution of PCR test results according to ages (month) in lambs and kids.

	Positive		Negative	
Lambs	N	%	N	%
≤ 1 month	18	36 %	0	0 %
2 months	15	30 %	0	0 %
3 months	16	32 %	1	2 %
Total	49	98 %	1	2 %
Kids	N	%	N	%
≤ 1 month	20	40 %	0	0 %
2 months	15	30 %	2	4 %
3 months	10	20 %	3	6 %
Total	45	90 %	5	10 %

Table 8. The distribution of PCR test results according to ORFV specific primers.

Primers	Primer Lettering	N Positive/%	N Negative/%	*P value*
GIF/IL-2	A	57 (57%)	43 (43%)	0.162
PPP1-PPP4	B	49 (49%)	51 (51%)	0.841
PPP3-PPP4	C	10 (10%)	90 (90%)	0.001
ATI gene primer	D	3 (3%)	97 (97%)	0.001
Orf 1-Orf 2	E	62 (62%)	38 (38%)	0.016
ORFV-B2L	F	41 (41%)	59 (59%)	0.072
ORFV-A32	G	30 (30%)	70 (70%)	0.001
vIL-10-3 vIL-10-4	H	49 (49%)	51 (51%)	0.841
OVA32 LF1 OVA32 LR1	I	24 (24%)	76 (76%)	0.001
VR1 VR2	İ	58 (58%)	42 (42%)	0.110
PPV JGR	J	-	100 (100%)	
ORFV 011-A	K	1 (1%)	99 (99%)	0.001
ORFV 059-A	L	4 (4%)	96 (96%)	0.001
ORFV 011-B	M	-	100 (100%)	
ORFV 059-B	N	17 (17%)	83 (83%)	0.001
ORFV 109	O	-	100 (100%)	
ORFV 110	P	1 (1%)	99 (99%)	0.001
ORFV 127	R	26 (26%)	74 (74%)	0.001
045 gene	S	24 (24%)	76 (76%)	0.001
B2L gene primer	T	33 (33%)	67 (67%)	0.001
F1L gene primer	U	27 (27%)	73 (73%)	0.001
Alpha-tubulin	Ü	69 (69%)	31 (31%)	0.001
From B2L gene primers	V	45 (45%)	55 (55%)	0.371
ORFV 011-C (B2L)	Y	3 (3%)	97 (97%)	0.001
ORFV 059-C (F1L)	Z	17 (17%)	83 (83%)	0.001

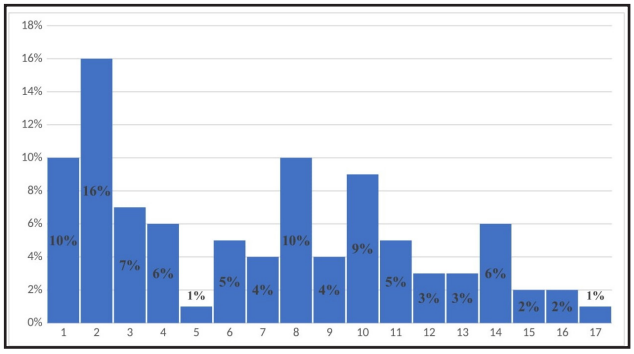

Figure 1. The graph the distribution of single / multiple primer groups and along with the percentage ratios of ORFV post the PCR test.

DISCUSSION

It was detected the presence of ORFV as 87.3% in imported breeds and 39.3% in domestic breeds, and they found high differences [11]. On the other hand, another researcher reported that the morbidity of ecthyma disease was 100% in 13 Gökçeada breed and 20 Maltese breed animals aged 9 months, and 97% in 29 Turkish Saanen breed animals of the same age [29]. In our study, high viral genome positivity was detected in local breed lambs (Sakiz) and kids (Kil goat). Furthermore, while statistically significant findings were achieved in the Sakiz and Anatolian Merino breeds in terms of GIF/IL-2 and B2L primers, no significant results were found in kids. We think that the reason for this is the large number of herds in the breeds owned by the public, lack of attention to hygiene conditions in the sheepfolds, and environmental conditions in the pastures of the animals.

It has been demonstrated in many studies that ORFV infection has no effect on gender [3,28]. Researchers reported that the reason why there was no relationship between the gender of the animal and the prevalence of the disease was that both gender groups in that region were kept under the same management system [28]. In this study, a higher percentage of ORFV genome presence was detected in female lambs and kids than in males. PPP1-PPP4 and ORFV-A32 primers showed statistically significant results in female lambs. However, no statistically significant findings were obtained in terms of gender in kids. Although a difference was detected in percentage, we recommend that detailed studies be carried out to obtain a significant difference, since the number of females and males in our lamb samples was not equal.

ORFV infection among young animals has very high morbidity and mortality rates [2,19]. It is stated that infectious ecthyma is likely to become a significant problem in animals that are young, immunocompromised, or housed in overcrowded, stressful environments [23]. In the current study, ORFV genome presence was detected more positivity in lambs and kids aged ≤ 1 month, than in those in the 2- and 3-month age group. Statistically significant findings were observed in ≤ 1 month old and 2-month-old lambs with GIF/IL-2 and B2L primers, and in ≤ 1 month old kids with Orf1-Orf2, vIL-10-3, vIL-10-4, and B2L primers.

In the study conducted in Bangladesh, 10 out of 13 samples were found to be positive. Researchers stated that other primers (vIL-10-3 vIL-10-4, OVA32LF1 OVA32LR1 and VR1 VR2) they found were closely related to GIF/IL-2 [3]. As a result of the research, GIF/IL-2 primer was found to be at very high levels. However, future sequence studies will determine which isolates it is related to.

In the current research, the PPP1-PPP4 primer was found at a high level in the samples, while the PPP3-PPP4 primer was found at a lower level. In Greece, found that 14 of 21 samples taken from 11 sheep, 8 goats, and 2 cattle were positive for the PPP1-PPP4 primer, and that 6 of the 7 samples that were negative were positive for the PPP3-PPP4 gene primer using semi-nested PCR [18].

In an investigation conducted in Nigeria, 100% positivity was found with the Orf1-Orf2 primer in 60 male goats [1]. In our research, a very high level of positivity for the Orf1-Orf2 primer was found. For this reason, we recommend focusing on more detailed research on the Orf1-Orf2 primer in our region.

In the research, 58 (58%) animals were found to be positive in the PCR analysis performed using the VR1-VR2 primer. In a study conducted on 8 goats in Gabon, Africa, 6 (75%) samples were determined to be positive with the VIR primer [25]. The investigators found positivity in the VIR1 VIR2 primers in 14 of 21 samples from 21 samples taken from 11 sheep, 8 goats and 2 cattle in their study in Greece [18]. They emphasized that it is necessary to obtain diagnostic results using different primers of ORFV in order to obtain more sensitive and specific results.

In this study, 26 animals (26%) were found to be positive in the PCR analysis performed using

the ORFV 127 primer from 100 samples. While the presence of this gene was determined in the light of the results obtained with the ORFV 127 primer in the study, less positivity was found in the results based on the ORFV 011 primers. However, no positivity for the ORFV 109 primer was detected. In Argentina, the presence of the ORFV 127 gene as nucleotides has been reported to be 3-5%. The researchers have stated that clearer interpretations can be made for the ORFV 011, 020, 109 and 127 genes as a result of the use of more molecular markers or the determination of the whole genome sequence due to the differences in nucleotide sequences and genes in the Argentine isolates, the evolution of the virus and the introduction of various viruses into the country [26].

In this study, 24 animals (24%) were determined to be positive in the PCR analysis performed using the 045 gene primer, and 69 animals (69%) were determined to be positive in the PCR analysis performed using the alpha-tubulin primer. In a study conducted by collecting 50 scab samples from lesions, occurring in the mouth area of sheep and goats in Iran was determined that 25 samples (50%) were positive in PCR analysis with the 045 gene primer [8]. Another researcher stated that 21 of 21 samples taken from 11 sheep, 8 goats and 2 cattle in Greece were detected positive for alpha-tubulin and 045 gene primers [18]. A different investigator in Italy between 2012 and 2014, they performed PCR analysis for the 045 gene in scab samples from a total of 32 animals, 5 goats and 27 sheep, and detected positivity in 28 of 32 samples (87.5%) [12]. In their investigation, they stated that ORFV infection developed in relation to the host rather than the specific characteristics of the virus. They also speculated that the 045 gene circulated in Southern Italy and may have come from animals exported from other countries. While our research determined the presence of 045 gene primers, the alpha-tubulin primer was found to be at the highest level among all primers. For the 1st time in Turkey, ORFV alpha-tubulin gene sequences were detected extensively in local breeds at the molecular level. We recommend that more detailed studies be conducted on this subject and the evaluation be investigated in depth.

In this investigation, 41 animals (41%) were determined to be positive in the PCR analysis performed using the ORFV-B2L primer. In one investigation, PCR results using the ORFV-B2L gene primer on 12 oral scab samples showed an 80% positive rate [27]. Samples of 55 domestic goats (34 adults and 21 cubs aged between 2 weeks and 4 months) were collected in the northern part of Egypt and were detected positive by PCR analysis for the ORFV-B2L gene primer. They could not detect a significant difference between the results of phylogenetic comparison with sequences from both neighboring (Sudan, Ethiopia and Israel) and Asian and European countries (Turkey, Malaysia, Taiwan, China and India). They also reported that the positivity of the ORFV-B2L gene, which they found by PCR, was caused by vaccine strains and wild strains due to intensive vaccination in the region.

In this study, 33 animals (33%) were found to be positive in the PCR analysis performed using the B2L gene primer. A group of researchers reported that positivity was detected in 3 out of 15 sheep (20%), 4 out of 15 goats (26.6%), and 1 out of 9 people (11.11%) in PCR analysis using the B2L primer [30]. Another group of researchers in a study conducted on a farm with 500 animals, found 24 of them positive (80%) in the PCR analysis performed with the B2L primer in 30 sheep showing ORFV clinical symptoms [10]. In Kashmir, they detected the presence of the B2L gene in 31 samples as positive (86.11%) in 36 samples of scab lesions in sheep and goats [2]. A team of scientists determined 3 samples -at the 1077 bp level- as positive (37.5%) in the PCR application performed with the B2L primer in 8 goats [20]. In India, it was identified 3 samples as positive for the B2L gene -at the 1137 bp level- [22]. The positive rate we obtained for the B2L gene by PCR is similar to that reported by Maganga *et al*. [20] was found to be close to the results. As a matter of fact, the base range of the B2L gene determined by electrophoresis was reported by Nagarajan *et al*. [22] was found to be similar.

In a study conducted in China by collecting serum samples from 349 goats, 225 samples were found to be positive (64.47%) with the ORFV 011-B2L primer [7]. In Eastern Sudan, 2 shell lesion scrapings were collected from each suspicious animal species in a herd of 87 camels, 51 sheeps and 13 goats, and in conventional PCR analyzes using the ORFV 011B2L gene primer, positive results were obtained in 21 samples [19]. Among the 100 samples used in our study, we found that 3 animals (3%) were positive as a result of PCR using the ORFV 011-C (B2L) primer. In our study, the positivity rate in the ORFV 011-C (B2L) gene primer was found to be significantly lower

than other studies on this subject. They stated that studies with high levels of positivity may be due to the transmission of the virus between sheep and goats, the development of cross-reactivity, genetic similarity in the hosts, and some breed sensitivities.

In this study, 17 (17%) animals were found to be positive by PCR using the ORFV 059-C (F1L) primer. In a study conducted in China by collecting serum samples from goats, positivity was determined with the ORFV 059-F1L primer [7]. Genetic analysis and histopathological research of ORFV with the F1L gene primer PCR method was performed using 50 sheep and goats in Iran. The results of histopathological findings were found to be 100% positive, and as a result of PCR analysis, 25 of 50 scab samples were found to be positive (50%) at the 1062bp level. These researchers believe that periodic vaccination for ecthyma infection is not performed in sheep and goats in Iran, and that the infection may have been transmitted from previously infected animals or soil/environment [9].

In this study, low positivity rates were detected in 4% for the ORFV 059-A primer, 3% for the ATI gene primer, 1% for the ORFV 110 and ORFV 011-A primers. Moderate positivity rates were found to be 17% for the ORFV 059-B primer, 24% for the OVA32LF1 OVA32LR1 primer and 27% for the F1L primer. The high positivity was found to be 3% as the ORFV A32 primer, 45% as the B2L gene primers, and 49% as the vIL-10-3/vIL-10-4 primer. Since these primers are newly studied primers in the last 10 years, we recommend that they be examined in more detail through sequence and phylogenetic studies in ORFV-infected animals in our country and their sources be determined.

No positivity was obtained in PCR analyzes using PPV JGR, ORFV 011-B and ORFV 109 primers in the 100 samples used in this study. However, we believe that it would be more appropriate to draw definitive conclusions by studying larger ORFV infected sheep and goat herds in our country.

CONCLUSIONS

In this study, with use of PCR applications, the presence of GIF/IL, PPP1-PPP4, Orf1-Orf2, VIR1 VIR2, vIL-10-3 vIL-10-4, B2L and Alpha tubulin primers was detected in terms of ORFV in the Burdur region. For this reason, we would like to point out that it is necessary to perform sequence and phylogenetic analyzes regarding the primers mentioned above. In this way, it will be possible to identify new wild isolates of ORFV in the Burdur region and our country and to form the basis for vaccine studies for these isolates. In our findings, we detected the groups with the highest number of binary ORFV primers in the tissues. It was also observed that Alpha tubulin and Orf1-Orf2 primer pair were included in most groups. We recommend that future studies focus primarily on these primers.

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***Ethical approval*.** All animals (housed inside the quarantine area of the pens) procedures performed in this study were approved by the "Bioethics Committee from the Department of Experimental Animals (Permit 21.10.2020/677)", at the Makü Hadyek, Burdur, Turkey.

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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