Kocatepe Veterinary Journal

Kocatepe Vet J. (2023):16(3):375-382

DOI: 10.30607/kvj. 1251239

RESEARCH ARTICLE

Updated Molecular Characterization of Orf Virus in Türkiye

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ABSTRACT

Orf (ORFV) virus is endemic in sheep and goats in Türkiye and detected with increasing incidence in Spring. Despite this significant prevalence rate of ORFV, a few molecular characterization studies have been conducted in Türkiye and all of them have focused on only B2L gene region so far. The aim of this study was to determine the molecular characterization of different gene regions of ORFV isolated from field studies. In the study, partial genome sequencing of different gene regions (B2L, F1L and VIR) of positive ORFV isolates detected by real-time PCR from seven sheep with suspected Orf in 2018-2020 were performed. Molecular characterizations were determined by bioinformatics studies. After the molecular and in-silico processes, we found remarkable diversification in the evaluation of each gene region. This result showed that different variants are more likely to have circulated in different parts of the Türkiye. When the study is evaluated as a whole, it is thought that the F1L gene region should be considered in addition to the B2L gene region in molecular studies. Molecular data of ORFV should be updated and followed to provide the best efficiency of the prevention and control strategy.

Keywords: Amino acid, molecular, orf virus, phylogeny, Türkiye

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Türkiye'de Orf Virusunun Güncellenmiş Moleküler Karakterizasyonu

ÖZ

Orf virusu Türkiye'de koyun ve keçilerde endemiktir ve ilkbaharda artan oranlarda tespit edilmektedir. ORFV'nun ülkedeki bu önemli prevalans oranına rağmen, Türkiye'de bugüne kadar birkaç moleküler karakterizasyon çalışması yapılmış ve hepsi sadece B2L gen bölgesine odaklanmıştır. Bu çalışmanın amacı saha çalışmalarında izole edilen ORFV'nun farklı gen bölgelerinin moleküler karakterizasyonlarının belirlenmesidir. Çalışmada, 2018-2020 yıllarında Orf şüpheli yedi adet koyundan real-time PCR ile izole edilen pozitif ORFV izolatlarına ait farklı gen bölgelerinin (B2L, F1L ve VIR) kısmi genom dizilemesi yapıldı. Biyoenformatik çalışmalar ile moleküler karakterizasyonları belirlenerek GenBank'ta bulunan izolatlarla ilişkileri değerlendirildi. Moleküler ve in-siliko çalışmalardan sonra, her bir gen bölgesinin değerlendirilmesinde dikkate değer bir çeşitlilik tespit edildi. Bu sonuçlar, farklı varyantların ülkenin farklı bölgelerinde dolaşıma girmiş olma olasılığının yüksek olduğunu göstermiştir. Çalışma bir bütün olarak değerlendirildiğinde, moleküler çalışmalarda B2L gen bölgesinin yanı sıra F1L gen bölgesinin de dikkate alınması gerektiği düşünülmektedir. Hastalıkla ilgili korunma ve kontrol stratejisinin en iyi verimini sağlamak amacıyla ORFV'nun moleküler verileri güncellenmeli ve takip edilmelidir.

Anahtar Sözcükler: Amino asit, filogeni, moleküler, orf virus, Türkiye

To cite this article: Kaplan M. Koç B.T. Pekmez K. Çağırgan A.A. Arslan F. Updated Molecular Characterization of Orf Virus in Türkiye. Kocatepe Vet J. (2023):16(3):375-382

Submission: 14.02.2023 **Accepted**: 13.09.2023 **Published Online**: 15.09.2023

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INTRODUCTION

Orf virus (ORFV) causes a significant disease, referred to as contagious ecthyma (CE), in different animal species (sheep, goat, etc.) and human. ORFV belongs to the genus of *Parapoxvirus*, a subfamily of *Chordopoxvirinae,* family of *Poxviridae* (ICTV 2020). ORFV is an epitheliotropic virus, and it causes proliferative lesions on the skin. Lesions are mostly local epitheliotropic; however, it may convert to a life-threating disease in case of complication with a secondary infection or occurrence on mouth, lips, nostrils, and nasal area (Spyrou and Valiakos 2015). Lambs are more sensitive than sheep and, the disease is more severe in the late summer and early fall. Although mortality is low, morbidity is approximately 90% (Fleming and Mercer 2007, Nettleton et al. 1996). The genome of ORFV is linear double-stranded DNA which is 139 kb in length. The genome has high GC content at the rate of nearly 65%; 88 genes are identified for ORFV genome and some of them are the forefront for molecular characterization (Delhon et al. 2004). *B2L* (ORFV011) is the essential gene region for the genetic and immunogenic characterization of ORFV (Abrahão et al. 2009, Hosamani et al. 2006). Similary, F1L (ORFV059) is another immunogenic gene of ORFV (Zhao et al. 2011). Besides these large immunogenic envelope genes, virulence genes were identified which were claimed important for pathogenesis and immune responses such as VIR (ORFV020), VEGF, vIL-10, GIF (Peralta et al. 2018). Considering the molecular characterization studies of orf virus in Türkiye, only the B2L gene region was studied. (Akkutay-Yoldar et al. 2016, Karakas et al. 2013, Şevik 2017 and 2019). Sheep and goat breeding is an important part of animal production in Türkiye. Significant efforts have been spent to improve through projects conducted in recent years. In this context, studies are carried out on many viral diseases including CE. CE is widespread in Türkiye, and especially affects the health of sheep and goat. There hasn't been much notification related to zoonotic cases of orf virus in Türkiye thus far. Different gene regions belonging to ORFV have not yet been characterized in Türkiye (Akkutay-Yoldar et al. 2016, Karakas et al. 2013, Şevik 2017 and 2019). The aim of this study was multiple comparisons using by three gene regions, B2L, F1L, and VIR, to better understand the current genetic circumstances. In this context, we performed phylogenetic analyses and amino acid comparisons for each gene region.

MATERIALS AND METHODS

Samples and preparation

In this study, samples were taken from sheep with suspected CE. Oral swab samples taken from six sheep were added to 2 ml PBS and vortexed, and the supernatant was collected and used for DNA extraction. Tissue samples (liver, spleen, lymph nodes, etc.) taken from a sheep were homogenized by homogenizer. The homogenate was centrifuged at 3500 rpm at +4 °C for 15 minutes, then the supernatant was collected and used for DNA extraction. Information of the samples including location, year, tissue type, isolate name and GenBank acces number are given in Table 1.

Table 1. The information about collected materials and relevant genes for PCR and their accession numbers from GenBank.

Province	Sample type	Date	Isolate name	Region	Accession no
				B2L	MW492036
Denizli	Swap	2018	TR/ORFV/2020/Bor1	F1L	MW492043
				VIR	MW492050
				B2L	MW492037
İzmir	Swap	2019	TR/ORFV/2020/Bor2	F1L	MW492044
				VIR	MW492051
				B2L	MW492038
Manisa	Internal organ	2018	TR/ORFV/2020/Bor3	F1L	MW492045
				VIR	MW492052
				B2L	MW492039
Manisa	Swap	2020	TR/ORFV/2020/Bor4	F1L	MW492046
				VIR	MW492053
				B2L	MW492040
Muğla	Swap	2018	TR/ORFV/2020/Bor5	F1L	MW492047
				VIR	MW492054
				B2L	MW492041
Uşak	Swap	2019	TR/ORFV/2020/Bor6	F1L	MW492048
				VIR	MW492055
				B2L	MW492042
İzmir	Swap	2019	TR/ORFV/2020/Bor7	F1L	MW492049
				VIR	MW492056

DNA Isolation and Real-Time PCR

Viral DNA extraction was performed using the Roche MagNA Pure LC 2.0 Instrument and the MagNA Pure LC Total Nucleic Acid Isolation Kit as recommended by manufacturer's instructions.

Real-time PCR was carried out using primers and probe previously described by Bora et al. (2011). The nucleotide sequence of the primers and probe are given in Table 2. Roche probe master PCR kit and thermal cycler (Roche LightCycler® 480) were used for DNA amplification. PCR was carried out in a 20 µl reaction volume containing 5µl template DNA, 0,75 µl of each primer (10 pmol), 0,5 µl probe (10 pmol), 10 µl LightCycler® 480 Probes Master and 3 µl PCR grade water. The PCR cycling conditions of the Real Time PCR were given in Table 2.

Table 2. Oligonucleotides used in real-time and conventional PCR and conditions in thermal cycler.

Method	Gene	Primers	Cycle	Reference
Real-Time	*Pol*	5 -TACACGGAGTTGGCCGTGATCTTGTA-3	Pre Den: 95 °C/5 min 40 Repeats: (95 °C/10 sec → 64 °C/45 sec → 72°C/1 sec)	Bora *et al.* (2011)
		5 -CGCCAAGTACAAGAAGCTGATGA-3		
		5 HexTGCATCGAGTTGTAGATCTCGCGGT-BHQ-1		
Conventional	B2L	5-ATGTGGCCGTTCTCCTCCATC-3	Pre Den: 95 °C/5 min 35 Repeats: (94 °C/30' → 58 °C/40'→ 72 °C/75')	Yang *et al.* (2014)
		5-TTAATTTATTGGCTTGCAGAACTCC-3		
	F1L	5-ATGGATCCACCCGAAATCACG-3	Pre Den: 95 °C/5 min 35 Repeats: (94 °C/30' → 60 °C/40'→ 72 °C/75')	
		5-TCACACGATGGCCGTGACCA-3		
	VIR	5-ATGGCCTGCGAGTGCGCG-3	Pre Den: 95 °C/5 min 35 Repeats: (94 °C/30' → 55 °C/40'→ 72 °C/60')	
		5-TTAGAAGCTGATGCCGCAG-3		

Sequencing PCR

Conventional PCR were conducted for each gene region (B2L, F1L, and VIR) according to the previous report (Table 2). A commercial kit (Xpert Fast Hotstart Mastermix with Dye) was used for amplification. The reaction mix with a total volume of 25 µl containing of 1 µl Forward and reverese primer (10 mM) 12.5 µl Fast PCR master mix, 5.5 µl water, 5 µl template DNA was prepared for each sample. Amplification conditions were given in Table 2. Amplified products were sent for sequencing in commercial lab (Macrogen, South Korea). Sanger dideoxy sequencing technique was performed and raw sequences belonging to each product were obtained. To correct errors, raw sequence histograms were edited in Tracer implemented in ClustalW method using the MEGAX software (Kumar et al., 2018). We checked the correspondence rates of similar sequences in BLAST, and confirmed.

Phylogenetic Tree and Molecular Characterization

We downloaded reference and genotypical sequences from GenBank. Clustal W algorithm in BioEdit software was used to align sequences. After alignment and trimming, "find best model" was used to determine parameters in MEGA X to be selected for phylogenetic trees. We constructed the phylogenetic trees for each gene region by performing the Maximum-likelihood method with 1000 bootstrap replicates. Kimura-2 parameter was used to calculate of the nucleotide similarities of the obtained sequences.

RESULTS

Positive seven ORFV samples detected by qPCR were also amplified for B2L, F1L, and VIR gene regions. After PCR processes, we viewed relevant amplicons in 1% agarose gel under blue-light transilluminator, which were 1137, 1023, and 552 base pairs for B2L, F1L, and VIR, respectively. These seven samples were uploaded to GenBank and accession numbers were provided for each corresponding sequence (Table 1). Three phylogenetic trees were generated based on three gene regions (Figure 1, 2A, 2B).

In B2L phylogenetic tree, our sequences were clustered in a clade, and they showed monophyletic relatedness to D1701 (HM133903), the reference sequence being the most prominent and essential for phylogenetic evaluation, reported from the USA. Furthermore, all previous ORFV sequences submitted from Türkiye showed diversities between each other and spread out into different clusters. Our sequences also were located far from the previous Turkish sequences in B2L phylogenetic tree (Figure 1).

In F1L phylogenetic tree, the strains that were sequenced for this study fell into three clusters.

TR/ORFV/2020/Bor5 and TR/ORFV/2020/Bor7 were clustered with reference strains belonging to China, which are Assam 2010 and Guizhou (KY412866 and KP057582, respectively). TR/ORFV/2020/Bor1, TR/ORFV/2020/Bor2, TR/ORFV/2020/Bor4, and TR/ORFV/2020/Bor6 were clustered with reference strains belonging to India and China which are referred to as Ludhiana, Jilin, and SY19 (KY412865, FJ808075, and MG712417, respectively). TR/ORFV/2020/Bor3 was located as the furthest strain from others and drew a distinct branch with a reference strain from Canada, named OV/Torino (AY040081) (Figure 2A).

VIR phylogenetic tree showed substantially similar demography with B2L phylogenetic tree. However, TR/ORFV/2020/Bor3, a strain in this study, fell into a distinct cluster located rather than our other strains. TR/ORFV/202/Bor3 was clustered with two reference sequences, which are SBF/Goabal-01 and SBF/Goabal-02 (KU672680 and KU672678, respectively), from a study conducted in Kashmir. TR/ORFV/2020/Bor1, TR/ORFV/2020/Bor2, TR/ORFV/2020/Bor4, TR/ORFV/2020/Bor5, TR/ORFV/2020/Bor6, TR/ORFV/2020/Bor7 were gathered at near of D1701 (HM133903), one of the oldest reference strain from the USA, as polyphyletic taxa as compared to TR/ORFV/2020/Bor3 (Figure 2B).

The identity and similarity matrix based on the nucleotide sequences of B2L showed that seven isolates of this study and previous Turkish sequences available in GenBank were high similarity ranging from 97.6% to 98.4%.

Basis on amino acid alignment, TR strains in this study interestingly corresponded to D1701 and similarity was 97%-99.8% to each three gene regions. The comparison of amino acid composition between reference and our strains revealed D1701 was the most corresponded strain rather than others.

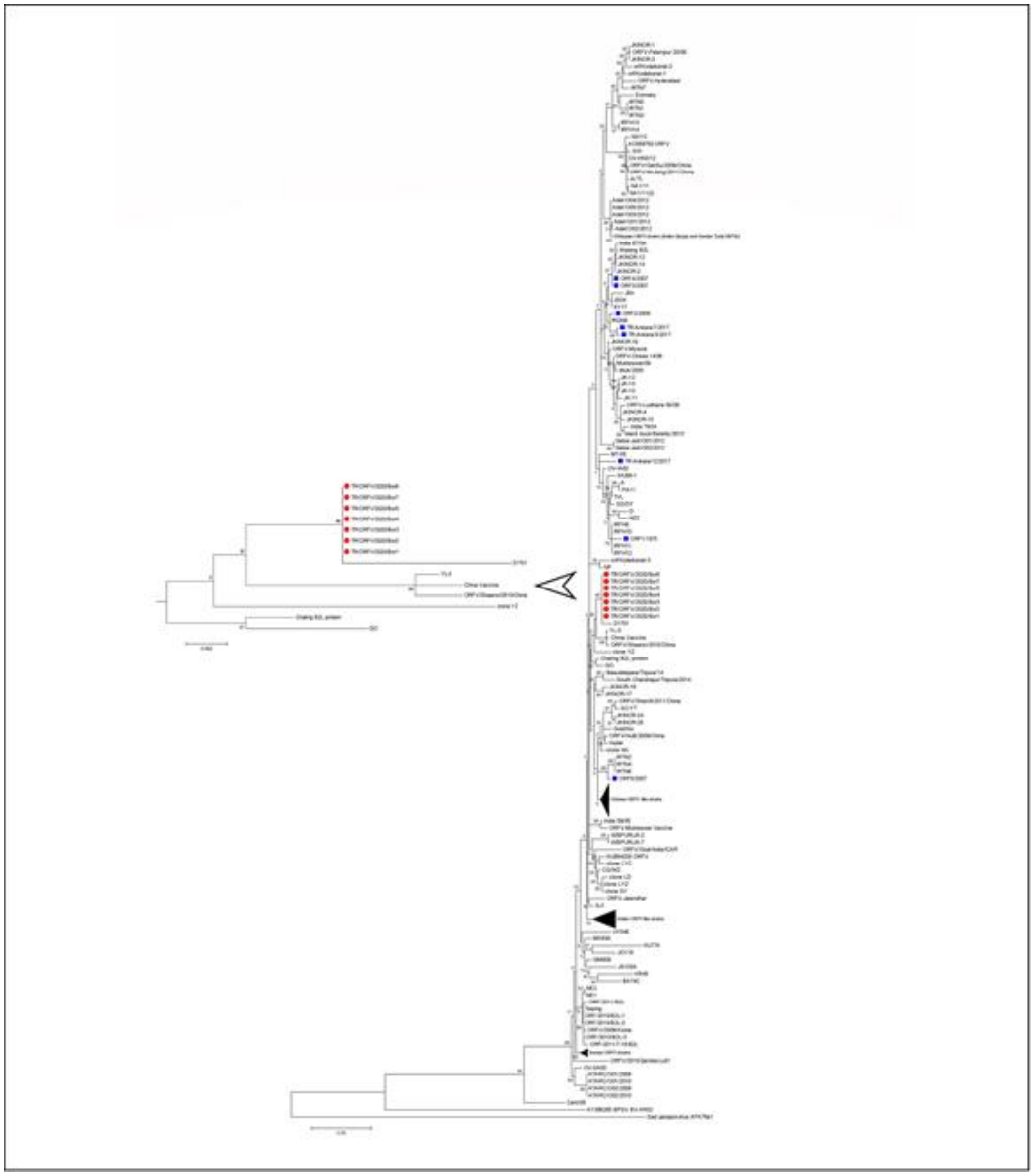

Figure 1: Phylogenetic tree based on the complete B2L gene region of ORFV. Turkish sequences in this study were marked with "●", and previous Turkish sequences belonging complete B2L gene region were marked with "■".

Figure 2: A- Phylogenetic tree based on complete F1L gene region of ORFV. B- Phylogenetic tree based on complete VIR gene region of ORFV. Turkish sequences in this study were marked with "●" in both trees.

DISCUSSION

Türkiye has close to 42 million sheep, making up a significant portion of the country's farm animal population, according to recently updated statistics data. (TUIK, 2020). ORFV is endemic in Türkiye among farm animals and primarily affects sheep and goat flocks (Akkutay-Yoldar et al. 2016, Karakas et al. 2013, Şevik 2017 and 2019). ORFV also has a zoonotic potential, and causes pustular dermatitis in humans mostly appears at the hands (Spyrou and Valiakos 2015). The lambing and shearing season, which lasts from April to June, is the most important risk factor for ORFV infection in both animals and individuals who closely contact with animals, such as farmers or veterinarians. (Spyrou and Valiakos 2015). Although ORFV is endemic in Türkiye, there haven't been enough molecular research to understand virus characterization. These few molecular studies have primarily focused on sequencing the B2L gene region to characterize ORFVs (Akkutay-Yoldar et al. 2016, Karakas et al. 2013, Şevik 2017, Şevik 2019). In this study, we sequenced the F1L and VIR gene sections in addition to the B2L gene region to better understand the molecular dynamics of ORFV in Türkiye. Thus, we presented a preliminary phylogenetic analysis using F1L and VIR (Figure 2) from sheep in Türkiye. Analyzing ORFV phylogenetic trees of this study, it was observed that our sequences were clustered almost similarly according to VIR (Figure 2) and B2L (Figure 1) gene region. However, tree constructed based on the F1L gene showed different demography. TR/ORFV/2020/Bor3 was on a separate branch as in the other trees. But, interestingly TR/ORFV/2020/Bor5 and TR/ORFV/2020/Bor7, located in the same branch, were in different cluster. This demography shows that F1L and B2L are more prominent in terms of molecular diversification and evolutionary assessment. Our findings compatible with previous phylogenetic research from throughout the world suggests that the B2L and F1L gene regions are better suited for phylogenetic analysis (Abdullah et

al. 2015, Peralta et al. 2018, Yang et al. 2015). Obtained data suggested it might be better particularly characterizing B2L and F1L genes in the next molecular investigations of ORFV.

In the assessment of three phylogenetic trees and the composition of amino acids belonging to relevant viruses, a vast majority of TR sequences in this study closely located monophyletic, as a sister group, to ORFV reference strain D1701 (HM133903). Strain D1701 was isolated from a sheep in Germany in 1975. However, the complete genome of D1701 has been available since 2011 in GenBank. Following owing to increasing biotechnological investigations, it has also become the biological platform for vaccines and drugs (McGuire et al. 2012). Cell culture-adapted variants D1701-B and D1701-V have been defined as immunomodulators for stimulation of the immune system, and/or viral vector platforms for delivering different microbial and viral antigens (Rziha et al. 1999 and 2016). TR strains in this study showed high homology to variants D1701-B and D1701-V. Therefore, if TR strains used in this study could be successfully isolated in cell culture, it might provide a novel molecular platform for local research.

Kumar et al. (2014) claimed to lead the host shifting with the existence of serine or glycine on the 249th amino acid of B2L. According to this prediction, if the 249th amino acid is serine, the host is more likely to be sheep, or if the 249th amino acid is glycine (G), the host is most likely to be the goat. As compatible with Kumar et al. (2014), the 249th amino acid of all TR ORFV in this study was serine (S) in this study.

In the Genbank database, sequences of B2L from Türkiye are limited, there are no sequences belonging to F1L and VIR or other gene regions. This fact makes it difficult to evaluate the molecular characterization outputs for Türkiye. A molecular study conducted in goats in the Central region of Türkiye in 2017 compared the amino acids by reference sequences available in GenBank (Şevik 2017). The author mentioned two amino acid substitutions (A134R and V309A) on B2L by comparison of other ORFV TR strains and claimed those substitutions might have occurred because of the host response (Şevik 2017). In another study which was performed in cattle in the same region in 2019, ORFV sequences showed 100% homology to ORFV goat sequences in 2017 (Şevik 2017 and 2019). In this study, we also compared amino acid substitutions on the alignment of all ORFV TR and some reference sequences available in the GenBank. Non-unique amino acid substitutions on several points of B2L already exist. Therefore, mentioned amino acid substitutions could not show what was the effect on virus-host interaction dynamics. It is thought that more detailed studies including larger and different gene regions or complete gene analysis of ORFV strains isolated from various animal species in Türkiye would benefit to a better understanding of the molecular dynamics.

F1L (ORF059), an immunodominant region of ORFV, has been less considered in the evaluation of its molecular dynamics when compared with B2L. We compared motifs of amino acids between reference and our sequences. Highly conserved gene regions of F1L are emphasized in some previous reports, which are KGD (Lys-Gly-Asp) motif, Cx3C motif, D/ExD motif, GAG motif, and KTR motif and completely corresponded to our recent sequences (Scagliarini et al. 2004, Yogisharadhya et al. 2018, Yu et al. 2020). On the contrary to other motifs, the proline-rich region might have affected phylogenetic diversification. Taken together, "PAPA-box" motif between 40th and 45th amino acids and point changes in amino acids might have led to TR/ORFV/2020/Bor5 and TR/ORFV/2020/Bor7 shift to distinct clade near Indian strains (Assam 2010 and Mukteswar Vaccine) (Figure 3). Yu et al. (2020), in the most recent study on amino acid motifs reported, mentioned that F1L is conservative, although the proline-rich region shows heterogeneity. Therefore, they claimed that the molecular characterization of B2L and F1L did not reflect the molecular dynamics of ORFV. However, the phylogenetic tree and amino acids of F1L in this study revealed it is necessary to be considered F1L for evolutionary dynamics.

VIR is one of the virulence genes of ORFV (Delhon et al. 2004, Hautaniemi et al. 2010, Peralta et al. 2018). Two arguments have been asserted on genetic variation of VIR so far. According to one of them, previously reported by Kottaridi et al. (2006), it was claimed that VIR had conservation in amino acid residues, and this did not associate with phylogenetic grouping based on host species, geographical origin, and time of isolation. A second argument by more recently reported by Peralta et al. (2018) mentioned that VIR and vIL10 were virulence factors for ORFV, and these were the most variable regions for all PPV genus viruses, including ORFV. In fact, our results were partially corresponded to these two arguments. TR sequences in this study except TR/ORFV/2020/Bor3 were 100% identical to D1701. TR/ORFV/2020/Bor3 was 98.4% identical to SBF/Goabal-02/Sheep Indian strain in 2016. This situation leads to think a number of variations coming from various geographic regions have already been circulating in the Western part of Türkiye.

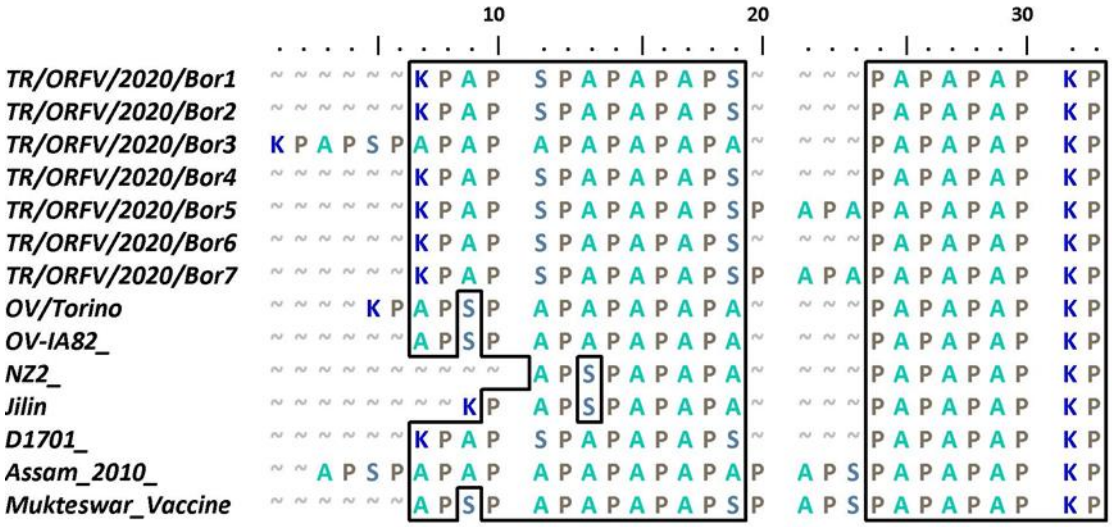

Figure 3: Comparison of residues on the proline-rich region (PAPA-box) of F1L in the alignment of the sequences.

CONCLUSION

In a conclusion, this study is a comprehensive molecular study conducted in three gene regions including B2L, F1L and VIR in recently isolated ORFVs in Türkiye. The study suggests that F1L gene region should be considered in phylodynamic evaluation besides B2L. According to results of the study, molecular knowledge of immunodominant genes might be verified by molecular analysis of virulence genes of ORFVs.

Türkiye is a geographical crossroad for many sectors from a global view. Livestock is highlighted in these sectors and affected from various situation. These clearly cause increasing circulation densities of viruses in the environment. The results exhibiting high homology between TR ORFV strains in this study and both European and Asian, suggested ORFV strains in Türkiye may likely originate from different geographies of the world.

Project Support Information: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical Approval: İzmir/Bornova Veterinary Control Institute, Animal Experiments Ethics Committee 16.12.2022, 2022/10 Number Ethics Committee Decision.

Conflict of Interest: The authors declared that there is no conflict of interest.

Author Contributions Rates: MK:%35, BTK:%35, KM: %10, AAÇ: %10, FA: %10

Acknowledgements: We thank the authorities of the Ministry of Agriculture and Forestry for providing us facilities to our scientific research.

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