AKADÉMIAI KIADÓ

Acta Veterinaria Hungarica

70 (2022) 2, 156–161

DOI:
10.1556/004.2022.00014

RESEARCH ARTICLE

Molecular evidence for concurrent infection of goats by orf virus and bovine herpesvirus 1

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Received: 25 March 2020 • Accepted: 25 May 2022
Published online: 28 June 2022

ABSTRACT

Orf is a disease of small ruminant animals, including goats and sheep, that is caused by a parapoxvirus. Although the mortality rate is low, economic losses may occur due to the clinical signs. Bovine herpesvirus 1 (BoHV-1) infection is known to cause respiratory and reproductive disorders mainly in cattle; however, it has been found to circulate among goats and sheep as well. In contrast to orf virus (ORFV), BoHV-1 does not induce clinical disease in goats. In this study, we aimed to detect the presence of ORFV by molecular methods and to uncover eventual simultaneous herpesvirus infections masked by orf disease signs. To this end, 82 goats, housed near to a cattle herd, were tested. By polymerase chain reaction (PCR), three goats (3.7%) were found to harbour both viruses, while an additional goat was positive for ORFV only. The PCR products were sequenced and phylogenetic analyses were performed. This study revealed that ORFV and BoHV-1 may be present simultaneously in an animal causing a concurrent infection. These data should be taken into consideration when looking for secondary pathogens in diseased goats, and the prevention methods should be developed accordingly.

KEYWORDS

goat, orf virus, bovine herpesvirus 1, concurrent infection, molecular characterisation

INTRODUCTION

Orf, also referred to as ecthyma contagiosum, is a significant disease that especially affects small ruminants and is clinically characterised by pustular dermatitis around the muzzle and nasal region (Spyrou and Valiakos, 2015). The causative agent, orf virus (ORFV) is an epitheliotropic virus with a zoonotic potential as it may cause substantially similar lesions on contacted skin regions in humans (Ginzburg and Liauchonak, 2017). ORFV is a member of the genus *Parapoxvirus* that belongs to the subfamily *Chordopoxvirinae* within the family *Poxviridae*. In ungulate animals, additional parapoxviruses occur such as the bovine papular stomatitis virus or the pseudocowpox virus. ORFV, like other parapoxviruses has an enveloped, ovoid-shaped virion and a large double-stranded DNA genome of more than 130 kilobase pairs (bp) in size. Among the more than 130 putative genes many encode structural proteins, one of which is the *B2L* gene. *B2L* is the major immunogenic envelope protein that is well conserved, therefore it is frequently utilised in immunological, molecular and phylogenetic studies (Sullivan et al., 1994; Delhon et al., 2004; Spyrou and Valiakos, 2015). Based on recent molecular phylogenetic analyses of the *B2L* gene, three main genogroups of ORFVs have been putatively established (Kumar et al., 2014; Şevik, 2017).

Bovine herpesvirus-1 (BoHV-1) belongs to the genus *Varicellovirus* of the subfamily *Alphaherpesvirinae* within the family *Herpesviridae*. Herpesviruses are also enveloped and have large double-stranded DNA genome consisting of a unique short and a unique long (UL) component. The genome encodes numerous structural proteins and among them different glycoproteins such as gB, gC, gD and gE. The gene *UL44* codes for glycoprotein C (gC), which is expressed in high amounts in the viral envelope and on the surface of the

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infected cells during the adsorption stage of the replication of BoHV-1 (Schwyzer and Ackermann, 1996; Muylkens et al., 2007). Thus, the gC protein is very important in terms of early diagnosis and prevention of the eventual secondary infections. In this regard, several molecular characterisation and genotyping studies have been performed based on the diversity of gC due to intensive virus–host interaction (Esteves et al., 2008). According to the latest genotyping studies, BoHV-1 strains are divided into five subtypes within three genogroups (BoHV 1.1, BoHV 1.2 and BoHV 5) (Esteves et al., 2008; Petrini et al., 2019).

BoHV-1 is a notorious viral agent that is able to infect heterologous hosts, and it negatively affects the respiratory and reproductive organs of large ruminants. It also consistently suppresses the immune system of animals by its latency mechanism (Schwyzer and Ackermann, 1996; Muylkens et al., 2007). It has not yet been clarified whether BoHV-1 induces specific clinical signs among goats, in spite of its presence having been reported by various researchers previously (Six et al., 2001; Yesilbag et al., 2003; Mahmoud and Ahmed, 2009; Baydın and Bilge-Dağalp, 2017). On the other hand, caprine herpesvirus (CpHV) is more specific, and the infection leads to a detectable clinical disease in goats (Suavet et al., 2016). In addition, it has been demonstrated in a recent study that goats might be the reservoirs and sources of cross-transmission of BoHV-1 (Gür et al., 2019).

Co-infection or multi-agent infection has been repeatedly observed among small ruminants housed together. Dual or concurrent virus infections among goats and sheep have been revealed by many studies to date (Kul et al., 2008; Toplu et al., 2012; Malik et al., 2011). Especially, mass housing and disrupted management trigger the circulation of many microorganisms, including viruses, and this negative condition basically causes respiratory and dermatologic diseases (Spyrou and Valiakos, 2015; Ginzburg and Liauchonak, 2017). In this perspective, ORFV and BoHV-1 are important since both viruses are able to infect heterologous hosts and are widespread among animals of various species, in addition to their ability to cause immunosuppression (Spyrou and Valiakos, 2015; Ginzburg and Liauchonak, 2017).

The first aim of this study was to provide laboratory confirmation by polymerase chain reaction (PCR) screening for ORFV and perform molecular characterisation based on the partial *B2L* gene sequences in a goat flock affected by clinical orf disease. The second aim was to investigate the potential occurrence of two respiratory viruses (CpHV and BoHV) by targeting the gene of gC in the above-mentioned flock showing also persistent upper respiratory tract infection and respiratory distress besides the ulcerative lesions of the gums.

MATERIALS AND METHODS

Animals

The samples originated from a family-owned livestock farm located in Kuyucak town of Aydin province, Turkey (37°55'56.7"N-28°29'23.1"E). In the farm, both cattle and goats were kept. The goat flock consisted of eighty-two animals, the majority of which had clinical signs such as nasal discharge, muzzle lesions and mild fever. Some goats had also ulcerative gingival lesions and respiratory disorders (Fig. 1). Scab materials and gingival/nasal swabs were collected by clinician veterinarians using an informed consent form, signed by the owner of the goats. No experimental animals were used in this study. All examinations on the animals were performed by veterinary clinicians.

Sample processing and viral DNA extraction

The scab material and the swabs were soaked in PBS, then homogenised to an approximately 10% suspension. After centrifugation at 9,000 *g* at 4 °C for 15 min, the supernatants were used for nucleic acid purification. The DNA was extracted by using the High Pure Viral Nucleic Acid Kit (Roche Life Sciences) according to the manufacturer's instructions.

PCR and sequencing

Detection of the presence of the three viruses (BoHV-1, CpHV-1, ORFV) was performed by PCR and sequencing. The amplification of a part from the gene of protein gC of CpHV-1 was attempted by the method described by Ros et al. (1999). For screening the presence of BoHV-1 in the samples, we used a PCR, targeting also the gene of gC. The sequences of the primers were modified from the method reported by Traesel et al. (2015). Finally, for the detection of ORFV, a semi-nested PCR with primers targeting the *B2L* gene was used (Inoshima et al., 2000). The sequence of the primers along with the expected size of the amplicon and the thermal conditions of the reactions for each virus are summarised in Table 1. The result of the PCRs was checked by agarose gel electrophoresis in 1% TAE buffer. The gels were photographed using a BLooK™ blue light LED transilluminator (GeneDireX Inc., Miaoli, Taiwan). The PCR products of the expected size were purified from the reaction mixture with the PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (MA, USA), then sent for DNA sequencing by Sanger's dideoxy method to a commercial service provider (BMLabosis Ltd., Ankara, Turkey).

Sequence analysis and phylogeny reconstruction

The raw sequences were edited using the Finch TV software (Patterson et al., 2004). The edited sequences were confirmed with the BLAST algorithm at the NCBI website. For phylogenetic analysis, reference sequences were downloaded from the GenBank. Multiple nucleotide (nt) alignments were made in the MEGA X software (Kumar et al., 2018) using the ClustalW algorithm. Phylogeny reconstruction was performed by using the Neighbour-Joining (NJ) method. The MEGA X was also used for model selection. For the ORFV and BoHV-1 gene fragments, the Tamura-Nei and Tamura-3 models were predicted, respectively. For calculating bootstrap values, 1,000 replicates were applied.

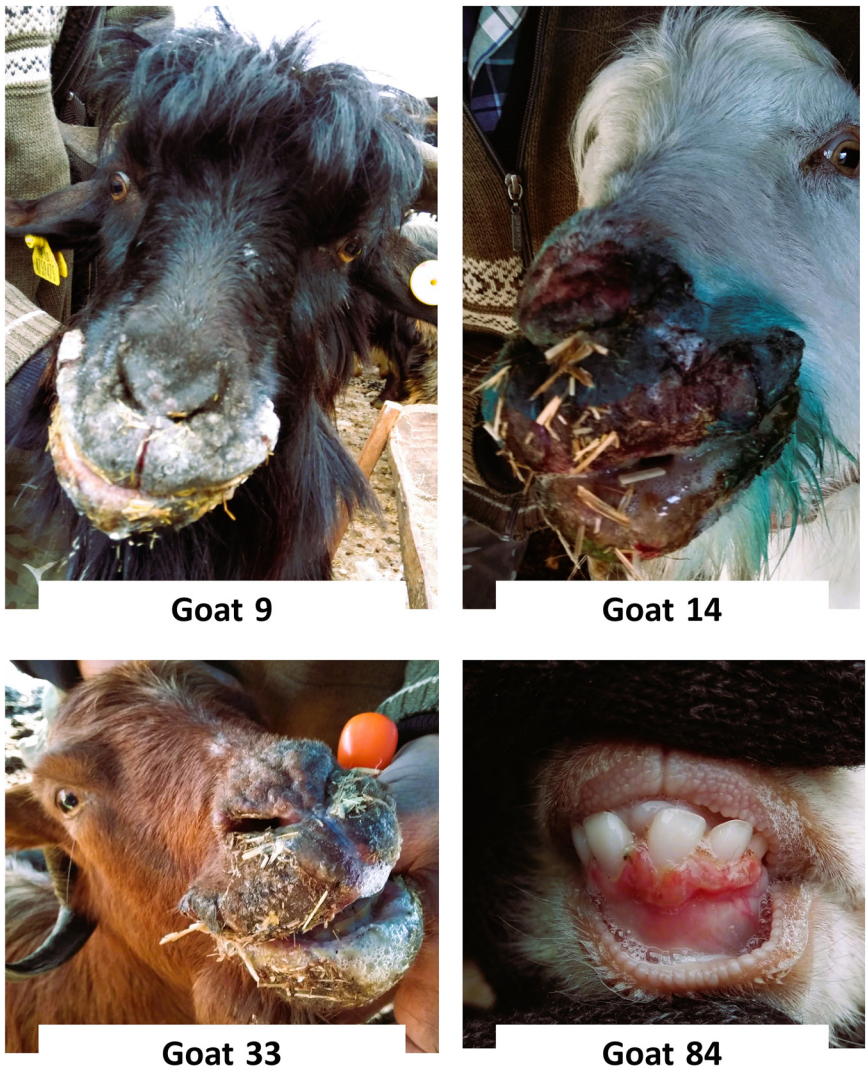

Fig. 1. Clinical picture of the muzzle of the four goats that tested positive for the presence of orf virus. Due to the exudative erosions, feed and litter straw adhere to the face and nose of the affected animals. Gingivitis is also evident

RESULTS

PCR and sequencing

No positive reaction was obtained with the PCRs intended for the detection of CpHV. The ORFV-targeting PCRs gave positive results with the samples of four goats (4.9%), which are marked as 9, 14, 33 and 84 in Fig. 1. After sequencing the PCR products, the identity of the fragments from the *B2L* gene was confirmed by the BLAST results. The sequences of sample 9 and sample 14 were identical, and shared over 98% identity with the other two sequences. Three out of the four ORFV positive animals (except No. 84) were found positive also for the presence of BoHV-1 using the PCR capable of detecting the gene of structural protein gC. Thus a dual infection condition was confirmed in three goats (3/82; 3.7%). The newly determined sequences were submitted to the GenBank and assigned accession numbers MK645800→03 (ORFV) and MK659886-88 (BoHV-1).

Sequence analysis and phylogeny reconstruction

The four partial *B2L* gene sequences were compared to their counterparts of other ORFVs retrieved from the GenBank. As the topology of the NJ tree (presented in Fig. 2A) shows, the sequences obtained in this study clustered in a separate, common branch within Cluster III of Group I. This branch is localised taxonomically as a sister branch of the previously submitted Chinese (KU884328) and Turkish (KC491193 and KP869116) ORFV sequences.

The authenticity of the newly determined BoHV-1 sequences was also confirmed by comparison to gC gene sequences of other BoHV-1 strains, retrieved from the GenBank. On the NJ phylogenetic tree (Fig. 2B), two sequences from goats 14 and 33 (marked as TR/BHV1/Goat 14 and TR/BHV1/Goat 33) grouped in a common cluster, including a reference strain, 'Cooper' within the BoHV-1.1 subtype. The third sequence (TR/BHV1/Goat 84) originating from goat 84, appeared on a separate branch close to strain 'SV/63-06' belonging to subtype BoHV-1.2 (Fig. 2B).

Table 1. Data of the PCRs including the sequence of the primers, the size of the expected amplicons and the thermal conditions of the reactions for the detection of the presence of the three viruses

Virus target gene	Primer sequences	Product length (bp)	D A E temperature/ duration (°C s^{-1})	Reference
Caprine HV gC	F: 5′-TGGCTGCTTGCGCTCGTCTG-3′ R:5′-GGGCGCCTCGTTGTCGTCGT-3′	349	ID: 95/300 95/30 62/30 72/60 35 cycles FE: 72/300	Ros et al. (1999)
Bovine HV-1 gC	F: 5′-TCGCGGCGCCAAGTGTACAC-3′ R: 5′-CGCGATGGGCGACCTAGCGGC-3	419	ID: 94/300 94/50 62/55 72/60 40 cycles FE: 72/300	Traesel et al. (2015) and this study
Orf virus *B2L* (semi-nested PCR)	F: 5′-GTCGTCCACGATGAGCAGCT-3′ *R: 5′-TACGTGGGAAGCGCCTCGCT-3′	1,134	ID: 95/300 94/30 55/30 72/60 35 cycles FE: 72/300	Inoshima et al. (2000)
	IF: 5′-GCGAGTCCGAGAAGAATACG-3′	594	ID: 95/300 94/30 50/30 72/60 35 cycles FE: 72/300	

HV: herpesvirus; D: denaturation step; A: annealing step; E: chain elongation step; ID: initial denaturation; FE: final elongation; F: forward; R: reverse; OF: outer forward; IF: inner forward; *primer used in both the first and second round of the semi-nested PCR.

DISCUSSION

The livestock industry in Turkey has 11 million goats according to the latest report of the Turkish Statistical Institute; therefore, goats occupy an important position in the production of meat and milk (TURKSTAT, 2018). However, infections negatively affect both the health and the yields of goats; particularly, persistent and/or chronic infections consistently cause problems. Investigation of these infections and application of control programs have huge importance in impeding economic losses.

This study presents data on dual infection by BoHV-1 and ORFV in a goat flock in Turkey. ORFV is a well-known and widespread viral agent of small ruminants all over the

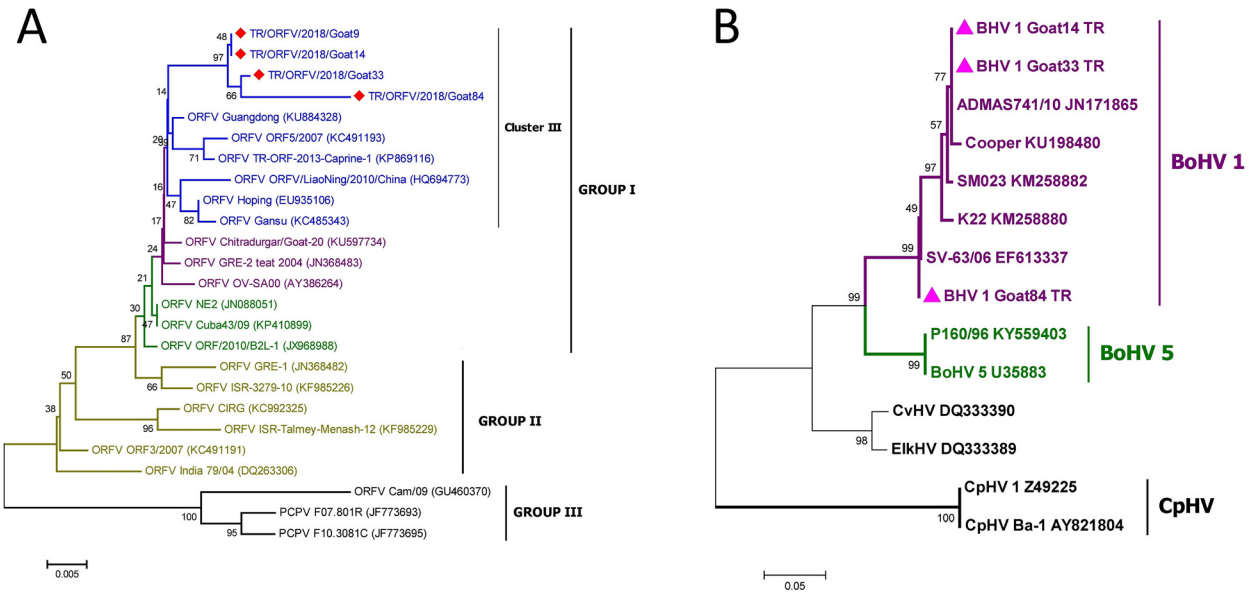

Fig. 2. Phylogeny inference of the newly detected viruses. A: Neighbour-joining tree calculated on partial *B2L* gene nucleotide sequences of orf viruses. The newly obtained sequences are marked with diamonds. Bootstrap percentage values as obtained from 1,000 resamplings of the data set are given at the nodes of the tree. The scale bar shows the distance corresponding to 0.005 substitutions per nucleotide position. B: Phylogenetic tree based on partial gC sequences of bovine herpesviruses (BoHVs). Caprine herpesviruses (CpHVs) were used as outgroup. The three novel BoHV-1 sequences, determined in this study, are marked with triangles. Bootstrap percentage values as obtained from 1,000 resamplings of the data set are given at the nodes of the tree. The scale bar shows the distance corresponding to 0.05 substitutions per nucleotide position

world (Spyrou and Valiakos, 2015). In Turkey, ORFV is enzootic, and occasionally triggers local outbreaks. Researchers have generally emphasised its economic impact and role as a predisposition factor for other diseases (Akkutay-Yoldar et al., 2016; Şevik, 2017, 2019). BoHV-1 is not a significant disease-causing agent for goats. Conversely, goats have been insinuated to play a critical role as a reservoir in transmission of BoHV-1 to cattle (Muylkens et al., 2007; Gür et al., 2019). However, ORFV and BoHV-1 should not be overlooked because of their potential suppressive effects on the immune systems of goats.

In this study, the ORFV positivity rate (4.9%) was found lower than expected according to the clinical observation of the goats in the farm we examined. The reason for this condition might be that the goats were already in convalescence. The ORFV infection in goats is usually investigated with serological tests in Turkey, thus, molecular data have been limited for detailed evaluation. The first ORFV sequences deposited from Turkish goats in the GenBank database originated from retrospective samples in 2013 (Accession no: KC491189-93, KF714235, KF714235; unpublished data). Since then, only two molecular prevalence studies have included a small number of relevant sequences (Akkutay-Yoldar et al., 2016; Şevik, 2017). In the global view, molecular studies on ORFV have not provided a formal genotyping yet which is accepted by the majority of authorities. However, some recent papers have presented informal genotyping in which three main genotypes have been usually exhibited in phylogenetic tree topologies (Kumar et al., 2014; Şevik, 2017). Likewise, sequences in this study formed a separate branch which was located near other Turkish strains and a Chinese ORFV strain in Genogroup I. The ORFV sequences in this study shared high identity rates between each other. The sequences derived from goat 9 and 14 were 100% identical, and shared 99.6 and 98.5% identity with the corresponding sequence from goat 33 and 84, respectively. This latter sequence (marked as TR/ORFV/Goat 84) occupied a relatively long branch within the new clade (Fig. 2A). Moreover, with the exception of TR/ORFV/Goat 9, BoHV-1 was detected in the other three ORFV positive goats.

There are a few reports on the existence of BoHV-1 in goats which have stated that keeping goats and cattle together has induced a predisposition for inter-species transmission of viruses (Muylkens et al., 2007; Gür et al., 2019). Likewise, a similar situation might have occurred in the farm examined by us. In some bovine samples obtained from animals with respiratory disease signs, the presence of BoHV-1 was detected. The nt sequence of the PCR products from the bovine samples was 100% identical (data not shown) with that of TR/BHV1/Goat 14, a finding which supports the possibility of cross-transmission. Gür et al. (2019) have implied that goats could be a reservoir for BoHV-1 infection. This study has already confirmed this claim, and also indicated that BoHV-1 might have caused mild infection in goats under variable conditions. Molecular studies on BoHV-1 in goats are scarce, and the majority of the performed studies have been based on serological techniques in Turkey (Yesilbag et al., 2003; Yesilbag and Güngör, 2009; Ataseven et al., 2010; Baydın and Bilge-Dagalp, 2017; Gür et al., 2019). Generally, seropositivity rates in goats to BoHV-1 have been reported to be between zero and 23% (Yesilbag et al., 2003; Yesilbag and Güngör, 2009; Ataseven et al., 2010; Baydın and Bilge-Dagalp, 2017; Gür et al., 2019). BoHV-1 serological studies in goats have been rarely performed, but two of them (Yesilbag et al., 2003; Yesilbag and Güngör, 2009) have reported 5.5 and 38.2% positivity in 2003 and 2009, respectively, from northwestern Turkey (the Marmara region). In a recent study, Gür et al. (2019) have detected 20.9% seropositivity in goats in inner western Anatolia. Although the molecular positivity rates for BoHV-1 in this study were close to the results of Yesilbag et al. (2003), the variation in the frequency of positivity draws the attention to the necessity of detailed studies with both serological and molecular virological tests.

According to our current knowledge, concurrent infection by ORFV and BoHV-1 in a goat flock has not been reported yet. Thus, our study is the first to report a potential synergistic interaction between the two viruses among goats. However, the existence of this interaction could not be claimed soundly due to the lack of data. For the full assessment about synergistic viral interaction, it should be confirmed by detailed virological, immunological, pathogenetic and experimental clinical analyses. As a further aim, ORFV and BoHV-1 should be investigated by molecular and serological techniques; thus, more comprehensive data could be provided on the prevalence of co-infection.

ACKNOWLEDGEMENTS

The author thanks the staff of the BMLabosis for their help with the sequence analyses and all molecular process supplies. The author also thanks the regional veterinarians for collecting material from the animals. This research was supported by the Aydin Adnan Menderes University (Project Number: VTF-20005).

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