Molecular detection and identification of ovine herpesvirus-2 in small ruminants, Sistan region, Iran

Arya Abdollahi[1,3], Dariush Saadati[2*], Mehdi Rasekh[3], Mohsen Najimi[4], Dariush Sargazi[5]

1- Department of Microbiology, Faculty of Medicine, Golestan University of Medical Sciences, Gorgan, Iran., ORCID ID: 0009-0000-4381-108X
2- Department of Food Hygiene, Faculty of Veterinary Medicine, University of Zabol, Zabol, Iran. ORCID ID: 0000-0001-9002-1507
3- Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Zabol, Zabol, Iran. Email: mrasekh@ut.ac.ir, ORCID ID: 0000-0003-1505-6640
4- Department of Pathobiology, Faculty of Veterinary Medicine, University of Zabol, Zabol, Iran. Email: najimi.mohsen@gmail.com
5-Chabahar veterinary office, Sistan and Baluchestan provincial veterinary service, Iranian veterinary organization, Chabahar, Iran Email: Dr.dariush69@yahoo.com

***Corresponding author:** Dariush Saadati,
Zabol, Bonjar Road, new campus of University of Zabol, Faculty of Veterinary Medicine, postal code: 9861335856
Tel: +985431232272
Fax: +985431232251
Email: Saadatdariush@uoz.ac.ir & saadatidariush@gmail.com

Abstract

Malignant catarrhal fever (MCF) is a systemic, sporadic, and fatal viral disease affecting susceptible hosts such as cattle, buffalo, and deer. Ovine herpesvirus-2 (OvHV-2) is one of the main causes of MCF in livestock. OvHV-2 infects sheep asymptomatically, playing a significant role in the virus circulation. In this study, the prevalence of OvHV-2 in sheep (n:48) and goats (n:52) was determined through molecular tests conducted in the Sistan region located in the north of Sistan & Baluchestan province, Iran. OvHV-2 POL and ORF75 Tegument Protein genes were detected in 15 out of 100 blood samples (sheep:8, goats:7) through Hemi-Nested PCR (15%). Sequence analyses show 100% identity to the isolations reports from Pakistan (MK852172), Brazil (OP121121), Turkey (MN419921), Egypt (ON952534), and India (OR762746). The molecular confirmation of ORF75 sequences and their high identity to global isolates emphasizes the genetic conservation of OvHV-2 strains. There was no significant relationship between the OvHV-2 virus prevalence and independent variables, including species, age, and gender of the animal and location of livestock. However, flock size was significantly associated with the prevalence of the virus. These findings indicate that OvHV-2 is circulating in the Sistan region, causing MCF. Since traditional livestock farming is common in the region, carrier species and susceptible hosts are kept together, which is a risk factor for virus transmission to susceptible hosts like cattle. This study is the first molecular investigation of OvHV-2 in small ruminants from the Sistan region, contributing essential data to the understanding of MCF dynamics in southeastern Iran. To control and prevent the disease, it is essential to consider separating different livestock species in the region.

Keywords: Goats, MCF, OvHV-2, Sheep, Sistan

1. Introduction

Malignant catarrhal fever (MCF) is a systemic, lymphoproliferative, and usually fatal disease that can infect a wide range of Artiodactyla species such as cattle, water buffaloes, deer, bison, and pigs(1). Currently, some members of the genus *Macavirus*, belonging to the *Gammaherpesvirinae* subfamily of *orthoherpesviridae* family (2), have been identified as causative agents of the disease. Ovine herpesvirus-2 (OvHV-2) and alcelaphine herpesvirus-1 (AlHV-1) are the most well-researched members of the genus, causing inapparent infection in sheep and wildebeests, respectively. Reservoir hosts can transmit viruses to susceptible animals, which may cause MCF (3). Direct contact between susceptible animals and asymptomatic reservoirs is the most common route of virus transmission(4). Therefore, mixed grazing systems may be a potential risk factor for viral transmission(4).

Sheep-associated malignant catarrhal fever (SA-MCF) is a fatal disease induced by OvHV-2, primarily affecting susceptible hosts such as cattle, water buffalo, and deer（2）. The virus is maintained in reservoir hosts, mainly sheep and goats, without causing clinical signs（3）. Transmission occurs mainly through aerosolized viral particles in nasal secretions, with lambs shedding the virus at high rates between 5-6 months of age(5). The prevalence of OvHV-2 varies geographically, influenced by husbandry practices and livestock density（6）. Mixed farming systems, where reservoir and susceptible hosts are kept together, significantly increase the risk of SA-MCF outbreaks（4）. Additionally, climate conditions, flock size, and cross-border livestock movements play crucial roles in virus circulation（7, 8）. The disease is economically significant due to its high fatality rate and lack of an effective vaccine（9）.

To date, cases of MCF occurrence have been reported in wild and domesticated animals in Iran(11–13). Sistan and Baluchestan province is situated in the south-eastern part of the country, Mixed farming is common in these districts, susceptible and reservoir hosts to the MCF are usually kept together. To best of our knowledge this is the first study of MCF prevalence in blood samples from reservoir hosts (sheep and goats) in Sistan region using polymerase chain reaction (PCR).

2. Materials & Methods

2.1. Study area and Sample collection

The blood samples were collected from randomly selected 21 traditional husbandry sites located in the Sistan region including Zabol, Zahak, Nimruz, Hamun, and Hirmand districts in north of Sistan and Baluchestan province over one year (Feb 2023-2024). Sheep and goats were kept together in the flocks, and cows may occasionally be included in the flocks. The age of the animals ranged from six months to three years. A total of 5 ml of blood samples were randomly collected through the jugular vein of sheep (n=48) and goats (n=52). The samples were collected from apparently healthy animals examined by approved veterinarians and questionnaire forms were filled out. Collected blood transferred into the anticoagulant tubes (EDTA) and transferred on ice to

the virology laboratory, of the veterinary faculty of the University of Zabol. The samples were stored at 4°C for further analysis.

2.2. Sample processing and DNA isolation

The samples were centrifuged at 1600 r/min for 15 minutes. The Buffy coat layer was removed and used for DNA extraction. DNA extraction was performed using SinaPure™ DNA extraction Kit (Tehran, Iran) according manufacturer instructions. The concentration (ng/ml) of the extracted DNA was measured using a Nano-Drop spectrophotometer. Subsequently the DNA samples were stored at -20°C until further use.

2.3. PCR method

Hemi-nested PCR was used to amplify the OvHV-2 POL gene as described by Flach et al. (14). In brief, the primer sets used were as follows:
POL1 (5'-GGC (CT)CA (CT)AA (CT)CT ATG CTA CTC CAC-3'),
POL2 (5'-ATT (AG)TC CAC AAA CTG TTT TGT-3') and
OHVPOL (5'-CCA AAA TGA AGA CCA TCT TA-3').
At the primary stage, primers POL1 and POL2 were used to amplify 386bp fragment. All PCR reactions, with a total volume of 50 µL, included 1 µM of each primer, 4 µL of template DNA, 25 µL of 2x Master Mix RED (Ampliqon, Denmark), and nuclease-free water to reach a total volume to 50 µL. The thermal cycling conditions performed by 95°C for 15 minutes for initial denaturation followed by 94°C for 1 min, 60°C for 1 min, 72°C for 1 min for 25 cycles, and 72°C for 10 min for the final extension step. A positive sample obtained from our study was verified by Sanger sequencing and used as a positive control while nuclease-free water was used for the negative control instead of the DNA. In the second stage, primers OHVPOL and POL2 were used for amplifying a 172bp fragment. 2 µL of primary amplification products were used as template DNA in the second stage. Thermal cycling conditions were same as the first stage except, 34 cycles were performed. 5 µL of secondary reactions were run in 1.8% agarose gel. Hemi-nested PCR was conducted on the positive samples of OvHV-2 POL gene to amplify the ORF75 tegument protein gene. The primer sets employed for this amplification were:
556(5′-AGTCTGGGTATATGAATCCAGATGGCTCTC-3′),
755(5′-AAGATAAGCACCAGTTATGCATCTGATAAA-3′) and
555(5′-TTCTGGGGTAGTGGCGAGCGAAGGCTTC-3′) that described previously (10).
For the primary amplifying a 422 bp fragment, primer 556 and primer 755 were utilized. for the next stage primer 556 and primer 555 were employed for amplifying a 238 bp fragment. The PCR conditions were same as those used for amplifying the OvHV-2 POL gene. The PCR products were then run on a 1.8% agarose gel.

2.4. Sequencing and phylogeny analysis

Three positive PCR products of the ORF75 tegument protein gene obtained from the second reactions were sent to Microsynth AG, Switzerland for the Sanger sequencing. These samples were randomly selected from sheep positive samples. A 238 bp fragment of the ORF75 tegument protein gene was sequenced using primer 555.

derived sequences and obtained sequences from GenBank were aligned by running the CLUSTAL_W method. The maximum likelihood method was used to construct the phylogenetic tree by MEGA 7 software (15). Group confidence was estimated using bootstrap involving 1000 replications. The sequences have been deposited in GenBank with PP824398, PP824399, and PP824400 accession numbers.

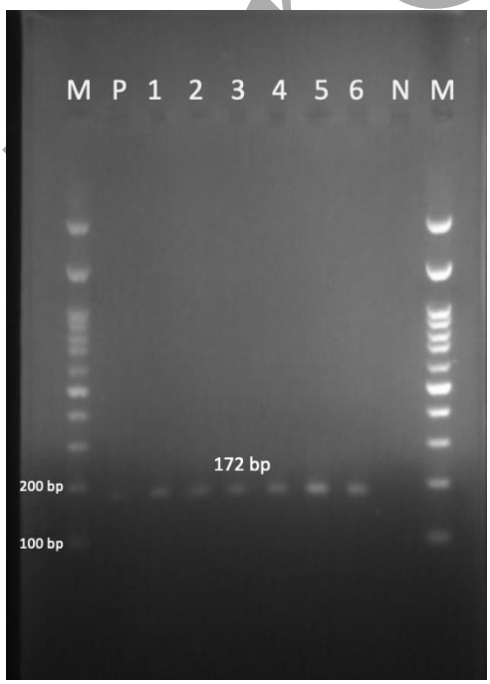
2.5. Statistical analysis

Statistical analysis was performed using SPSS version 25. The overall prevalence of OvHV-2 infection was calculated with a 95% confidence interval. The associations between infection prevalence and independent variables (species, age, gender, flock size, location) were assessed using logistic regression to calculate odds ratios and P-values. A significance level of $P<0.05$ was applied.

3- Results

3.1. Prevalence and risk factors of OvHV-2

In this study among 100 sampled animals, 15% (n:15) (95% CI (8.6%-23.5%)) were positive for OvHV-2 using hemi-nested PCR for detecting OvHV-2 POL and ORF75 tegument protein gene as recommended by WOAH(16). The prevalence of infection with the virus in sheep and goats were 16.7% (8/48) and 13.5% (7/52), respectively. The prevalence of OvHV-2 infection did not differ significantly between sheep and goats (P=0.654). Three samples collected from lambs less than one year were identified as positive for OvHV-2. The virus was identified in all districts of Sistan; however, the prevalence in the Zabol and Nimruz districts was lower compared to others. Also, there was no significant relationship between the OvHV-2 virus prevalence and age, gender and location of livestock. However, flock size was significantly associated with the prevalence of the virus as shown in Table 1.

A: 

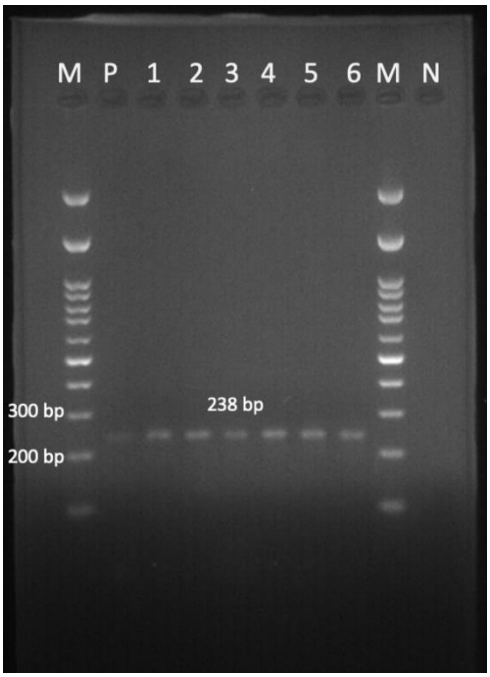
B: 

Fig 1. Hemi-nested PCR result from second stage reactions: (A) 172bp band of POL gene of ovine herpesvirus-2, (B) 238bp of ORF75 tegument protein gene of ovine herpesvirus-2. M: 100bp ladder, P: positive control sample, 1-6: positive samples of OvHV-2, N: negative control

Table 1
The number and prevalence of infection with ovine herpesvirus-2 by independent variables in 100 sheep and goats in Sistan region

Variables	Levels	Sheep Positive	Goats Positive	Total Positive	Odds Ratio	Significance*
Age of animal	Less than one year	25% (2/8)	9% (1/11)	16% (3/19)	1.08	P=0.915
	More than one year	15% (6/40)	15% (6/41)	15% (12/81)	1	Reference group
Gender of animal	Female	23% (8/35)	11% (4/35)	17% (12/70)	1.86	P=0.365
	Male	0% (0/13)	18% (3/17)	10% (3/30)	1	Reference group
flock size	Small	12% (3/26)	14% (4/29)	13% (7/55)	0.24	P=0.031
	Medium	6% (1/16)	8% (1/13)	7% (2/29)	0.12	0.020
	large	67% (4/6)	20% (2/10)	38% (6/16)	1	Reference group
Location of flock	Zabol	0% (0/11)	8% (1/12)	4% (1/23)	0.95	P=0.974
	Hamun	27% (4/15)	15% (2/13)	21% (6/28)	5.73	P=0.120
	Zahak	50% (2/4)	20% (2/10)	29% (4/14)	8.40	P=0.072
	Hirmand	14% (1/7)	33% (2/6)	23% (3/13)	6.30	P=0.130
	Nimruz	9% (1/11)	0% (0/11)	5% (1/22)	1	Reference group

* P-values are based on univariable logistic regression analysis. For each variable, different levels were compared with the reference group.

In the univariable logistic regression, only the flock size showed a significant association with OvHV-2 infection.

3.1. Molecular and Phylogenetic analysis

3 samples of 15 positive samples were sequenced and indicating 100% identity to each other using BLAST (Basic Local Alignment Search Tool) of the NCBI (National Center for Biotechnology Information). Furthermore, BLAST results show 100% identity to the isolations reports from Pakistan (MK852172), Brazil (OP121121), Turkey (MN419921), Egypt (ON952534) and India (OR762746). Also > 99% similarity were obtained with Mongolia (LC203437), India (MK059980) and Russia (P669276), as well as the RefSeq reference genome (NC007646). The phylogenetic analysis of the sequences obtained in this study shows close similarities to other sequences of the ORF75 tegument gene. Whereas obtained sequences (PP824398, PP824399, and PP824400) compared to other sequences show distance to the India (DQ229939), Turkey (JN991056), Germany (HM216478) and Brazil (KC123170).

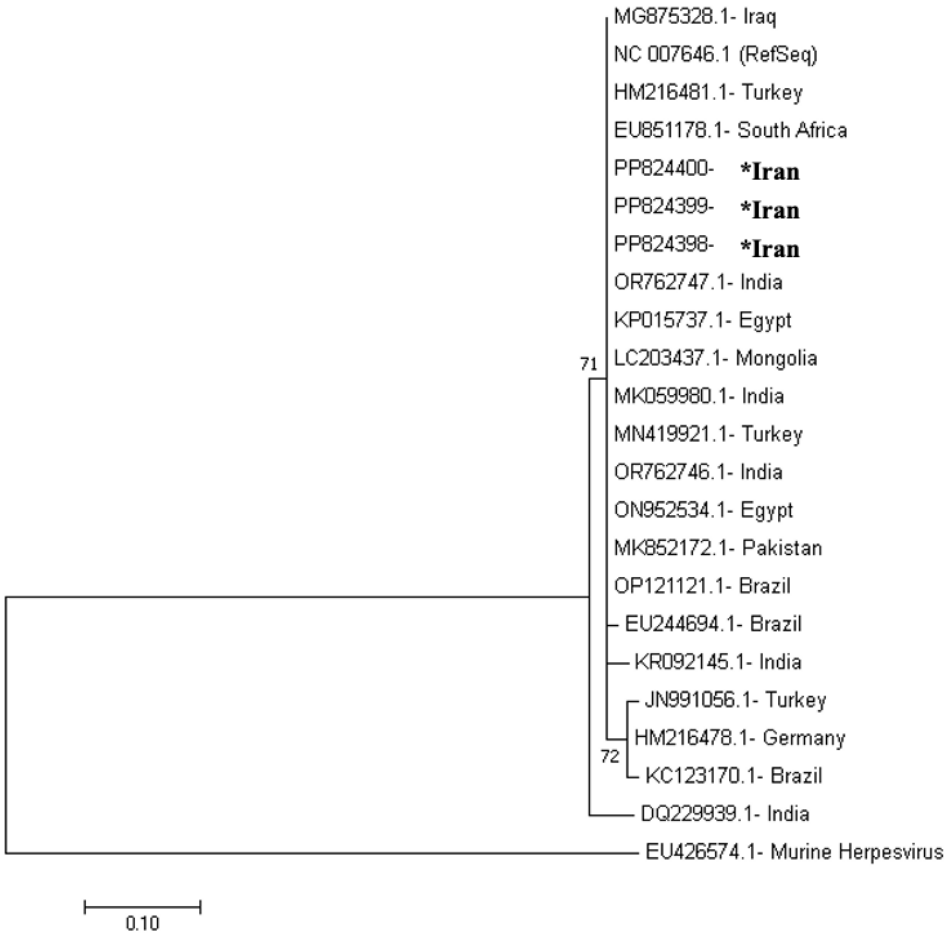


Fig 2. Phylogenetic tree of ORF75 tegument protein gene of sequences derived from this study (bolded) and deposited sequences in GenBank. The maximum likelihood method was used for tree construction by supporting 1000 bootstrap replications.

4. Discussion

In the present study, the overall prevalence of OvHV-2 was 15%, with specific rates of 16.7% in sheep and 13.5% in goats, as determined by Hemi-nested PCR. Our findings indicated that flock size is a significant factor in OvHV-2 infection in the Sistan region. It was observed that large flocks had higher infection rates compared to medium and small flocks, which could be due to increased interactions among animals in large-sized flocks, facilitating virus transmission. However, the difference in infection rates between medium and small flocks was not statistically significant.

The prevalence of OvHV-2 in sheep and goats in the Sistan region was found to be lower than that reported in other parts of Iran and neighboring countries(7, 8, 13, 17, 18). This discrepancy could be attributed to regional differences, including variations in climate and husbandry practices. Reductions in small ruminant flock sizes may paradoxically influence the epidemiological dynamics of OvHV-2.

There have been several reports on the prevalence of OvHV-2 in Iran and neighboring countries. According to Momtaz *et al.*(2009) and Seyfi Abad Shapouri *et al.*(2014), the prevalence of OvHV-2 in healthy sheep in central and western Iran was 100% and 82%, respectively, using PCR methods(12, 17). In a study on wild ruminants in Iran, Hemmatzadeh *et al.*(2016) reported 31% positivity for OvHV-2 using PCR(11). In Pakistan, prevalence rates were reported as 48% in sheep and 44% in goats in 2021(8), while Pekmez *et al.*(2022) found a 34% prevalence in sheep in Turkey using PCR on blood, organs, and aborted fetuses(18). Additionally, Khudhair *et al.*(2020) reported a prevalence of 100% in sheep and 86.6% in goats in Iraq's Al-Qadisiyah province(7).

Goats are natural hosts of caprine herpesvirus 2 (CpHV-2) (1). However, as we detected OvHV-2 in this study, some evidence indicates that goats are reservoir hosts for OvHV-2 (8, 12, 19), because mixed farming is common in this region, OvHV-2 may be transmitted between sheep and goats without any specific clinical signs. However, some studies have suggested that OvHV-2 can lead to subclinical infection in goats as well as exhibit clinical signs, such as fever and central nervous system disorders, such as ataxia and head tremors(20).

The Detection of positive samples in the lambs in this study corroborates the findings of Li *et al.*(1998), who reported that OvHV-2 could be transmitted horizontally to lambs in the flock and shed the virus at 5-6 months old (5). Additionally, Li *et al.* (2001) indicated that OvHV-2 can infect fetuses during pregnancy(19), as reported by Pekmez *et al.* (2022) (18).

Based on the results of BLAST and phylogenetic analysis, the sequences obtained in this study showed approximately 98–100% similarity to other published sequences of the ORF75 tegument protein gene internationally. These findings support other researchers findings that there is no major difference between OvHV-2 sequences globally(6-8). Besides, the inclusion of the RefSeq sequence (NC007646) in the phylogenetic analysis, highlights the significant similarity with the obtained sequences (> 99%). confirming high genetic conservation of the virus.

To the best of our knowledge, this is the first report to indicate OvHV-2 prevalence in sheep and goats and to confirm the presence of the disease in the Sistan region, southeast Iran. The prevalence of OvHV-2 was significantly lower in small and medium sized flocks compared to large flocks. Given that most flocks in the Sistan region are currently small sized due to prolonged drought conditions. This could be the reason for the low prevalence of the infection in this region. Considering trade relations across borders with neighboring countries, especially Pakistan, on the border of the study area, the consequences of legal and illegal movement of livestock within international borders should be considered by authorities. Because of the difference in OvHV-2 prevalence according to reports in different locations, these movements could potentially affect virus circulation.

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Ethics

The present research is an observational study. The protocol of this study was reviewed and approved by the Ethics Committee of University of Zabol with ethical code number IR.UOZ.REC.1403.001. The experiments were performed on sheep and goat blood samples. Blood sampling was done with the consent of the livestock owners and the identity of the owners remained confidential.

Conflict of Interest

The authors have no relevant financial or non-financial interests to disclose.

Authors' contribution

Acquisition of data, Analysis and interpretation of data, Drafting of the manuscript; A.A.
Study concept and design, Analysis and interpretation of data, Drafting of the manuscript, Statistical analysis, Study supervision; D.S.
Study concept and design, Study supervision; M.R.
Administrative, technical, and material support, Critical revision of the manuscript for important intellectual content; M.N.
Administrative, technical, and material support; D.S.

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Data Availability

The datasets generated and/or analyzed during the current study are not publicly available due to privacy concerns and restrictions related to livestock owners' confidential information. However, aggregated or general data may be made available from the corresponding author upon reasonable request via email.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors used ChatGPT (GPT-4, OpenAI) to assist with improving the language and enhancing the clarity and readability of the text. All content generated using this tool was carefully reviewed and edited by the authors, who take full responsibility for the final version of the manuscript.

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