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Research Paper

Molecular Detection and Identification of Ovine Herpesvirus-2 in Small Ruminants, Sistan Region, Iran

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ABSTRACT

Introduction: Malignant catarrhal fever (MCF) is a fatal disease affecting susceptible livestock species. Ovine herpesvirus-2 (OvHV-2), the main causative agent of sheep-associated MCF, establishes asymptomatic infections in sheep and goats, which serve as reservoir hosts. This study investigated the molecular prevalence of OvHV-2 in small ruminants from the Sistan region of southeastern Iran.

Materials & Methods: Blood samples were collected from 100 clinically healthy animals, including 48 sheep and 52 goats, from traditional husbandry systems in the Sistan region. DNA extracted from buffy coat samples was analyzed using heminested chain reaction (PCR) targeting the OvHV-2 POL and ORF75 genes. Selected positive samples were sequenced and subjected to phylogenetic analysis. Associations between infection and potential risk factors were evaluated using logistic regression.

Results: OvHV-2 DNA was detected in 15% (15/100) of the animals, including 16.7% (8/48) of sheep and 13.5% (7/52) of goats. No significant association was found between infection and species, age, sex, or geographical location. However, flock size was significantly associated with virus prevalence ($P<0.05$). Sequence analysis of the ORF75 gene showed 100% identity with isolates previously reported from Pakistan, Brazil, Turkey, Egypt, and India, indicating high genetic conservation.

Conclusion: OvHV-2 is circulating among sheep and goats in the Sistan region and may contribute to the risk of MCF in susceptible livestock. This study provides the first molecular evidence of OvHV-2 infection in small ruminants from the region and highlights the importance of management practices, particularly separation of reservoir and susceptible species, for disease control.

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1. Introduction

Malignant catarrhal fever [MCF) is a systemic, lymphoproliferative, and usually fatal disease that infects 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 the orthoherpesviridae family [2], have been identified as the 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 unapparent 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 represent a significant potential risk factor for viral transmission [4]. Sheep-associated MCF (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 and is 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]. Polymerase chain reaction (PCR)-based molecular techniques are widely used for the diagnosis and epidemiological investigation of OvHV-2 infections [10]."

To date, several 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, where mixed farming is common in these districts, susceptible and reservoir hosts of 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 the Sistan region using PCR.

2. Materials and Methods

2.1. Study area and sample collection

Blood samples were collected over a one-year period (February 2023–2024) from 21 randomly selected traditional husbandry sites across the Sistan region including Zabol, Zahak, Nimruz, Hamun, and Hirmand districts in northern Sistan and Baluchestan Province. In these flocks, sheep and goats were kept together, with cows occasionally be included. The animals ranged in age from six months to three years. A total of 5 mL of blood samples (sheep: n=48; goats: n=52) were randomly collected through the jugular vein. The samples were collected from apparently healthy animals, as confirmed by approved veterinarians, and relevant data were recorded using structured questionnaire. All blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes and transferred on ice to the virology laboratory at the Faculty of Veterinary Medicine, University of Zabol. The samples were subsequently 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 subsequently removed and used for DNA extraction. Total DNA was extracted using SinaPure™ DNA extraction Kit (Tehran, Iran) according manufacturer's instructions. The concentration (ng/mL) of the extracted DNA was measured using a NanoDrop spectrophotometer. The DNA samples were then stored at -20 ˚C until further analysis.

2.3. PCR method

A Hemi-nested PCR was used to amplify the *OvHV-2 POL* gene, following the protocol described by Flach et al. [14]. Briefly, 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, the primers POL1 and POL2 were used to amplify a 386bp fragment. All PCR reactions, with a total volume of 50 µL, contained 1 µM of each primer, 4 µL of template DNA, 25 µL of 2x Master Mix RED (Ampliqon, Denmark), and nuclease-free wa-

ter to reach a total volume to 50 µL. The thermal cycling conditions consisted of an initial denaturation 95 ˚C for 15 minutes, followed by 94 ˚C for 1 minute, annealing at 60 ˚C for 1 min, and extension at 72 ˚C for 1 minute, for 25 cycles, with a final extension step at 72 ˚C for 10 minutes.

A positive sample, previously verified by Sanger sequencing in our study, was used as a positive control, while nuclease-free water was used for the negative control instead of DNA. In the second stage,the primers OHVPOL and POL2 were used to amplify a 172bp fragment. For this stage, 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 that 34 cycles were performed. Subsequently, 5 µL of the 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′-AGTCTGGGTATATGAATCCAGATG-GCTCTC-3′),

755(5′-AAGATAAGCACCAGTTATGCATCT-GATAAA-3′) and

555(5′-TTCTGGGGTAGTGGCGAGC-GAAGGCTTC-3′) that described previously [10].

For the primary amplification of a 422- bp fragment, primer 556 and primer 755 were utilized. In the subsequent stage, primers 556 and 555 were employed to amplify 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 from the second reactions(randomly selected from positive sheep samples) were submitted to Microsynth AG, Switzerland for Sanger sequencing. 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 under accession numbers PP824398, PP824399, and PP824400.

2.5. Statistical analysis

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

3. Results

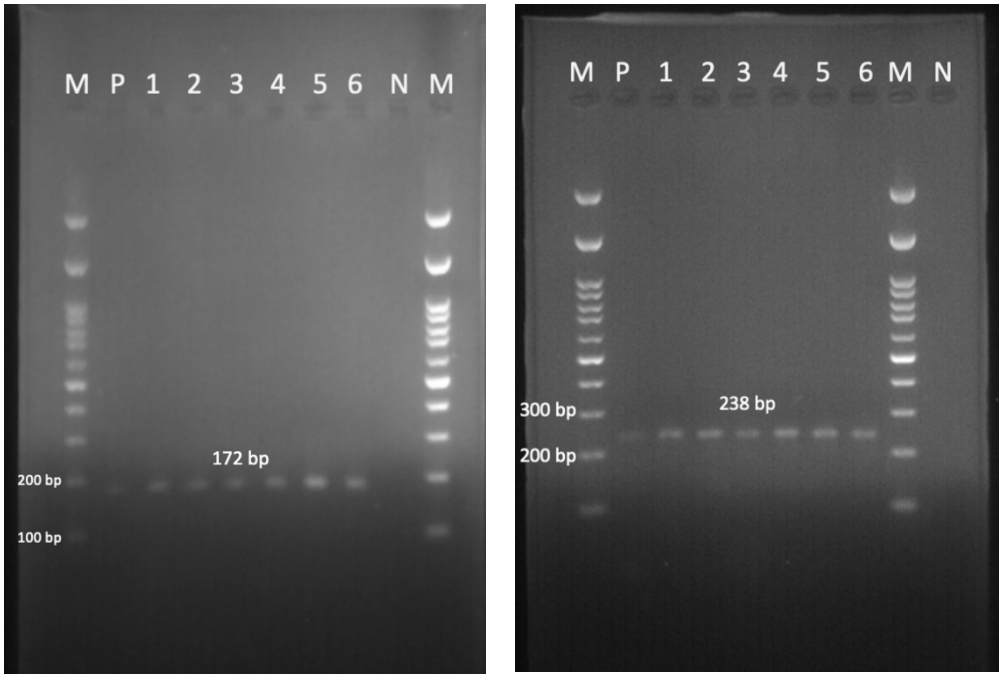
3.1. Prevalence and risk factors of Ovhv-2

Among 100 sampled animals, 15% (n:15) (95% CI (8.6%, 23.5%) were positive for OvHV-2 using hemi-nested PCR detecting the OvHV-2 POL and ORF75 tegument protein gene, as recommended by World Organisation for Animal Health (WOAH) [16]. Representative hemi-nested PCR amplification products of the OvHV-2 POL and ORF75 genes are shown in Figure 1. The prevalence of infection with the virus in sheep and goats was 16.7% (8/48) and 13.5% (7/52), respectively. There was no significant difference in the prevalence of OvHV-2 infection between sheep and goats (P=0.654). Three samples collected from lambs less than one year were identified as positive for OvHV-2. While the virus was identified in all districts of Sistan; prevalence in the Zabol and Nimruz districts was lower compared to others. Statistical analysis revealed no significant relationship between the OvHV-2 virus prevalence and the age, gender, or location of livestock. However, flock size was significantly associated with the prevalence of the virus, as shown in Table 1.

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

3.2. Molecular and phylogenetic analysis

Three out of the fifteen positive samples were sequenced, and BLAST (Basic Local Alignment Search Tool) analysis through the NCBI (National Center for Biotechnology Information) indicated 100% identity among them. Furthermore, BLAST results showed 100% identity to isolates reported from Pakistan (MK852172), Brazil (OP121121), Turkey (MN419921), Egypt (ON952534), and India (OR762746). Additionally, >99% similarity was observed with sequences



A B

Figure 1. Hemi-nested PCR result from second stage reactions

A) 172bp band of *POL* gene of OvHV-2, B) 238bp of ORF75 tegument protein gene of OvHV-2.

Note: M: 100bp ladder, P: Positive control sample, 1-6: Positive samples of OvHV-2, N: Negative control.

from Mongolia (LC203437), India (MK059980) and Russia (P669276), as well as the RefSeq reference genome (NC007646). Phylogenetic analysis of the sequences obtained in this study shows close similarity to other global ORF75 tegument gene sequences. However, when compared to specific isolates,the obtained sequences (PP824398, PP824399, and PP824400) exhibited genetic divergence from certain strains reported in India (DQ229939), Turkey (JN991056), Germany (HM216478) and Brazil (KC123170). The phylogenetic relationships between the sequences obtained in this study and reference OvHV-2 isolates are presented in Figure 2.

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 identify flock size as a significant factor in OvHV-2 infection in the Sistan region. Specifically, large flocks exhibited higher infection rates compared to medium and small flocks. This correlation could be due to increased interactions among animals within 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 notably lower than that those reported in other parts of Iran and neighbouring countries [7, 8, 13, 17, 18]. This discrepancy could be attributed to regional variations, including climatic conditions and husbandry practices. Paradoxically, fluctuations or reductions in small ruminant flock sizes may also influence the epidemiological dynamics of OvHV-2.

Numerous studies have documented the prevalence of OvHV-2 in Iran and its neighbors. 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) identified a 31% positivity for OvHV-2 using PCR [11]. In Pakistan, prevalence rates reached 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 foetuses [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]. While goats are the primary natural hosts of caprine herpesvirus 2 (CpHV-2) [1]), our detection of OvHV-2 in

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

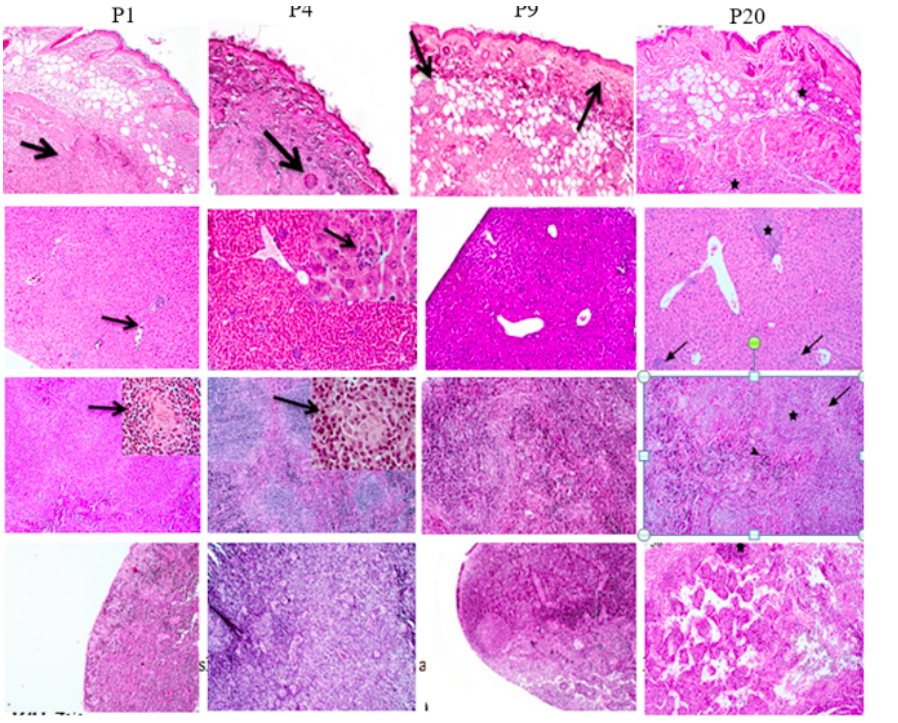
Variables	Levels	%			Odds Ratio	Sig.*
		Sheep Positive	Goats Positive	Total Positive		
Age of animal	Less than one year	25 (2/8)	9 (1/11)	16 (3/19)	1.08	P=0.915
	More than one year	15 (6/40)	15 (6/41)	15 (12/81)	1	Reference group
Gender of animal	Female	23 (8/35)	11 (4/35)	17 (12/70)	1.86	P=0.365
	Male	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.

goats supports growing evidence that they also serve as 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 in addition to exhibiting clinical signs, such as fever and central nervous system disorders, including ataxia and head tremors [20].

The detection of positive samples in the lambs during this study corroborates the findings of Li et al. (1998), who reported that OvHV-2 could be transmitted horizontally within flock with lambs shedding 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 BLAST results and phylogenetic analysis, the sequences obtained in this study exhibited 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 the high genetic conservation of the virus.

To the best of our knowledge, this is the first report to document the prevalence of OvHV-2 in sheep and goats and to confirm the presence of the disease in the Sistan region, southeast Iran. A key finding was that 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 may explain the lower prevalence of the infection in this region. Considering the cross-border trade and livestock movement with neighboring countries, especially Pakistan, the consequences of both legal and illegal livestock transport within international borders should be closely monitored by authorities. Because of the difference in OvHV-2 prevalence according to reports in different locations, these movements could potentially affect virus circulation.



e 6: Pathologic of skin tissues, liver, spleen and lymph nodes on day 30, A: Skin, B: Liver, C Spleen, D: Lymph node

Figure 2. Phylogenetic tree of ORF75 tegument protein gene of sequences derived from this study (bolded) and deposited sequences in GenBank

Note: The maximum likelihood method was used for tree construction by supporting 1000 bootstrap replications.

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Compliance with ethical guidelines

The present research is an observational study. The protocol was reviewed and approved by the Ethics Committee of University of Zabol, Zabol, Iran (Code: IR.UOZ.REC.1403.001). All procedures were performed on sheep and goat blood samples with the informed consent of the livestock owners, and all personal identities remained strictly confidential.

Data availability

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

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Authors' contributions

Conceptualization and study design: Dariush Saadati; Data acquisition: Arya Abdollahi; Analysis, data interpretation, and writing the original draft: Arya Abdollahi and Dariush Saadati; Statistical analysis: Dariush Saadati; Supervision: Dariush Saadati and Mehdi Rasekh; Review and editing: Mohsen Najimi; Project administration, technical, and material support: Mohsen Najimi and Dariush Sargazi.

Conflict of interest

The authors declared no conflict of interest.

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