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Molecular detection and characterization of ovine herpesvirus-2 using heminested PCR in Pakistan

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ABSTRACT

Background: Malignant catarrhal fever (MCF) is a highly fatal lymphoproliferative disease of cattle, deer, bison, water buffalo, and pigs caused by the gamma-herpesviruses alcelaphine herpesvirus-1 (AlHV-1) and ovine herpesvirus-2 (OvHV-2).
Objectives: This study aimed to determine the prevalence of OvHV-2 in sheep, goats, cattle, and buffalo in Rawalpindi and Islamabad, Pakistan, by applying molecular and phylogenetic methods.
Methods: Blood samples were aspirated from sheep (n = 54), goat (n = 50), cattle (n = 46) and buffalo (n= 50) at a slaughterhouse and several farms. The samples were subjected to heminested polymerase chain reaction (PCR), followed by sequencing and phylogenetic analysis of the OvHV-2 POL gene and the OvHV-2 ORF75 tegument protein gene.
Results: The highest percentage of MCF positive samples was in sheep (13%), whereas goat, cattle, and buffalo had lower positive percentages, 11%, 9%, and 6.5%, respectively. Four OvHV-2-positive PCR products obtained from sheep samples were sequenced. The sequences obtained were submitted to the NCBI GenBank database (MK852173 for the POL gene; MK840962, MK852171, and MK852172 for the ORF75 tegument protein gene). Phylogenetic analysis revealed a close similarity of study sequences with those of worldwide samples.
Conclusions: This study is the first cross-sectional study on the prevalence and molecular detection of OvHV-2 in apparently healthy cattle and buffalo that could be carrying OvHV-2 acquired from OvHV-2-positive sheep and goats. The results indicate that OvHV-2 is circulating in Pakistan. Further studies are needed to characterize OvHV-2 and elucidate further its prevalence.

Keywords: Malignant catarrhal fever; DNA Viruses, Nested Polymerase Chain Reaction, Ruminants, Pakistan

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INTRODUCTION

The Pakistani livestock sector contributes approximately 11% of Pakistan's annual gross domestic product and provides employment to approximately 62% of the population in rural areas when subsidiary industries are considered. The Pakistan national livestock herd comprises 29.6 million cattle, 27.3 million buffalo, 53.8 million goats, and 26.5 million sheep. Infectious diseases that affect animals within the national herd affect food chain security and the broader economy [1].

Malignant catarrhal fever (MCF) is an acute, systemic, usually lethal lymphoproliferative disease of cattle and even-toed ungulates, including pigs, deer, bison, and water buffalo [2-5]. MCF may be caused by any of the identified gammaherpesviruses that comprise the *Macavirus* classification group. The two most well-studied *Macavirus* members are ovine herpesvirus-2 (OvHV-2) and alcelaphine herpesvirus-1 (AlHV-1), which are maintained asymptomatically in sheep and wildebeest reservoir populations, respectively [6,7]. Reactivation of these viruses from latency in asymptomatic reservoir populations and subsequent transmission of infectious virus to susceptible Ariodactyla species may result in MCF development. Although aerosol OvHV-2 transmission to susceptible animals has been reported to occur up to about 5 km from where reservoir animals are located [8,9], the most common transmission route is most likely direct contact on mixed grazing grounds of susceptible species with nasal secretions of reservoir animals containing the infectious virus.

Clinically, MCF is characterized by fever, excessive salivation, and nasal and ocular discharges. Lesions are usually present on the buccal cavity and muzzle. Enlargement of lymph nodes is also a characteristic sign of MCF. Post-mortem lesions include the presence of petechial hemorrhages on the buccal cavity mucosa and in the gastrointestinal and respiratory tracts [9,10]. Symptoms of MCF in susceptible species are similar to those of other similar diseases, such as vesicular stomatitis virus, foot and mouth disease, and rinderpest [6,11]. Clinically affected susceptible hosts do not shed infectious viruses and are considered dead end hosts [12,13].

MCF cases have been documented in India [14] and Iran [15,16], which border Pakistan. In Pakistan in most rural areas, livestock farms, animal hospitals, and research centers, it is common to practice mixed farming, thereby keeping MCF reservoirs and susceptible animals in close proximity [17,18]. Therefore, it is likely that the lack of documented cases of MCF in Pakistan is a result of underreporting due to a lack of testing and awareness among veterinarians. Herein, for the first time in Pakistan, polymerase chain reaction (PCR) testing of blood samples derived from an abattoir and several farms were used to investigate the prevalence of OvHV-2 in cattle, buffalo, sheep, and goats in the Rawalpindi and Islamabad districts.

MATERIALS AND METHODS

Samples collection
Blood samples were collected from sheep (n = 54), goat (n = 50), cattle (n = 46), and buffalo (n = 50). Animal ages ranged between 4 months and 5 years. Blood samples were randomly collected from a slaughterhouse (Sihala slaughterhouse, Rawalpindi) and farms of the Rawalpindi and Islamabad districts, Pakistan. The animals in the slaughterhouse were from different cities and towns in the vicinity of District Rawalpindi. Blood was collected in

ethylene diamine tetraacetic acid (EDTA)-containing tubes and transported to the Virology Laboratory of the Department of Parasitology and Microbiology, Faculty of Veterinary and Animal Sciences, Pir Mehr Ali Shah, Arid Agriculture University, Rawalpindi for processing. On arrival at the laboratory, the blood samples were stored at 4°C.

Ethics statement
All the sampling was carried out under strict ethical conditions, with the permission of the animal owners. Animals were handled, and samples were taken by Pakistan Veterinary Medical Council certified professional veterinary clinicians/practitioners (Doctor of Veterinary Medicine). Experiments were performed in accordance with the Ethics Committee for Use of Animal & Human Subjects, Arid Agriculture University guidelines. All methods, including animal assessment and sample procurement, were performed without harm to the animals. No anesthesia, euthanasia, or any kind of animal sacrifice was part of this study.

Processing of samples and DNA isolation
Blood samples were centrifuged at 1,500 r/min for 5 min, and the buffy coat layer was removed carefully and stored in separate tubes at −20°C for subsequent DNA extraction. DNA isolation was performed using 100–120 µL from the buffy coat layer, and extraction was performed using the PureLink Genomic DNA Kit (Invitrogen). The extracted DNA concentration in the samples was measured using a Nano-Drop spectrophotometer. Extracted DNA samples were stored at −20°C.

Construction of the OvHV-2 positive control
A section of the polymerase gene (POL gene) was amplified from DNA extracted from BJ1035 cells [19] by using Qiagen's DNA blood and tissue kit (Qiagen, UK). Regions of the POL gene were amplified in accordance with the primers specified by Baxter et al. [20]. The amplified product was purified using Qiagen's PCR purification kit (Qiagen), in accordance with the manufacturer's protocol, and cloned into Topo vector PCR2.1+ (Life Technologies, UK). The purified DNA was used as a positive control in OvHV-2 POL gene PCRs.

Heminested PCR to amplify OvHV-2 POL and ORF75 tegument genes
Heminested PCRs were performed to amplify fragments of the OvHV-2 POL gene present in the extracted DNA samples. The primer sets used were [21]:
Primer POL1: 5′-GGC(CT)CA(CT)AA(CT)CTATGCTACTCCAC-3′,
Primer POL2: 5′-ATT(AG)TCCACAAACTGTTTTGT-3′, and
Primer OHVPOL: 5′-AAAAACTCAGGGCCATTCTG-3′.

DNA samples along with positive and negative controls were selected for PCR. Primer POL1 (forward) and Primer POL2 (reverse) were used in the first step to achieve primary amplification. To perform PCR, *Taq* 2X Master Mix (NEB, UK) was used. Approximately 1 µg of the extracted DNA was used in a total volume of 50 µL reaction mixture for PCR. Thermal cycling conditions were carried out with one cycle of 95°C for 15 min followed by 25 cycles of 94°C for 60 sec, 60°C for 60 sec, and 72°C for 60 sec, with a final extension at 72°C for 10 min. For the negative control, nuclease-free water was used. For secondary amplification, primers OHVPOL (forward) and POL2 (reverse) were used. Samples of 4 µL of each primary amplification product were placed in PCR tubes, and 46 µL of master mix was added to each tube. Cycling conditions for the secondary PCR were the same as for the primary PCR, except that 30 cycles of amplification were used. After amplification, 10 µL of each second PCR reaction were run on 1.8% agarose gel.

Heminested PCRs to amplify a fragment of the OvHV-2 ORF75 tegument protein gene were also performed on the DNA samples positive for the OvHV-2 POL gene. The primer sets used were [20]:

Primer 556: 5′-AGTCTGGGTATATGAATCCAGATGGCTCTC-3′
Primer 555: 5′TTCTGGGGTAGTGGCGAGCGAAGGCTTC-3′
Primer 755: 5′-AAGATAAGCACCAGTTATGCATCTGATAAA-3′

Primer 556 (forward) and 755 (reverse) were used to amplify a 422bp fragment for the primary amplification step of the PCR. Primer 556 (forward) and Primer 555 (reverse) were used in the secondary amplification step to amplify a 238 bp fragment. In both amplification steps, the PCR and electrophoresis conditions were the same as those used for heminested PCR of the POL gene.

Sequencing and phylogenetic analysis

Four OvHV-2-positive PCR products were sent for gene sequencing to Macrogen Korea. All four PCR products were from DNA samples taken from sheep. Of the four PCR products, one was from the POL gene PCR and three were from the ORF75 tegument protein gene PCRs from the second amplification step. Primers OHVPOL and POL2 were used to sequence the POL gene PCR product, and primers 556 and 555 were used to sequence the ORF75 tegument protein gene PCR product. Sequences derived from this study and those obtained from the GenBank database were aligned by applying the CLUSTAL_W method contained in the software Seaview. The distances were computed mean-wise and overall using MEGA7. The gene sequences were translated using Seaview. Sequences were subsequently analyzed using neighbor-joining to construct the phylogenetic tree [18]. The statistical significance of the relationships obtained was determined by bootstrap resampling analysis with 1,000 repetitions.

The sequences were deposited in the GenBank database. Accession numbers assigned to the sequences by the NCBI were: MK852173 for OvHV-2 POL gene and MK840962, MK852171, and MK852172 for the OvHV-2 ORF75 tegument protein genes, The NCBI accession number for the amino acid sequences for the POL gene was QDG03185 and those for the ORF75 tegument protein were QDC27829, QDG03183, and QDG03184.

RESULTS

Two hundred blood samples were taken from four different species (46 cattle, 50 buffalo, 50 goats, and 54 sheep) from different farms, including small household level holdings and a slaughterhouse in the Rawalpindi and Islamabad districts of Pakistan, using a convenient sampling method. All animals were apparently healthy at the time of sampling. DNA was isolated from the buffy coat of each of the centrifuged blood samples, and the extracted DNA was subjected to heminested PCR using primers recommended by the OIE for the detection of OvHV-2 [20,21]. Initial screening of the samples was carried out using primers targeting the virus POL gene. A plasmid containing the region of the OvHV-2 POL gene, targeted by the POL primers derived from BJ1035 cells (an OvHV-2 immortalized cell line) was used as a positive control. The samples exhibiting 172 bp bands (amplicons) were considered positive. No amplicon was observed in the negative control samples (**Fig. 1A**). The positive DNA samples were further tested for amplification of a region of the ORF75 tegument protein gene (**Fig. 1B**). Out of 200 samples, 79 were determined to be positive for both OvHV-2 POL and ORF75 tegument genes using heminested PCR (**Table 1**). The highest percentage of positive
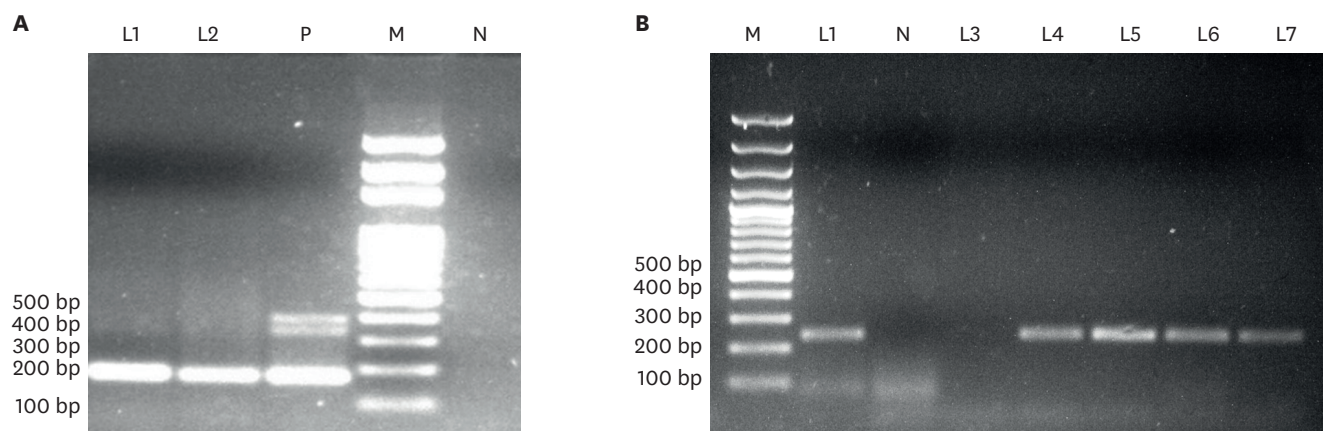
Fig. 1. Heminested polymerase chain reaction results showing (A) approximately 172 bp bands of the ovine herpesvirus-2 POL gene and (B) 238 bp bands of the ovine herpesvirus-2 ORF75 tegument protein gene.
L1 and L2, samples showing positive results; P, positive control; M, 100 bp ladder; N, negative; control L1, L4-L7, samples showing positive results.

Table 1. Number (%) of ovine herpesvirus-2-positive samples among the species sampled in the Rawalpindi and Islamabad districts, Pakistan

Species No.	Species	No. of samples	Ovine herpesvirus-2-positive samples	Percent of total (n = 200)
1	Cattle	46	18	9
2	Buffalo	50	13	6.5
3	Goat	50	22	11
4	Sheep	54	26	13

samples was in sheep (13%), whereas positive percentages for goat, cattle, and buffalo samples were 11%, 9%, and 6.5%, respectively (**Table 1**).

Molecular phylogenetic analysis of regions of the POL and ORF75 tegument protein genes of OvHV-2

The nucleotide sequences of the POL gene and ORF75 tegument protein gene regions of four OvHV-2-positive samples were compared with seven sequences and ten sequences, respectively, that had been reported by other researchers from around the world. Phylogenetic tree construction was based on aligned and retrieved NCBI sequences that were closely related and showed 99%–100% similarity with our sequences (**Fig. 2A and B**).

The phylogenetic analysis demonstrated that the POL gene sequence obtained in the present study (accession number: MK852173) clustered most closely to OvHV-2 isolates from HM216472 and AF327831 (Germany) (**Fig. 2A**). The isolated sequence showed more distant relationships with isolates of caprine herpesvirus-2 (KJ867526) and rupicapra pyrenaica gammaherpesvirus 1 (KP260923).

The phylogenetic analysis of the three ORF75 tegument protein gene sequences obtained in the present study (MK840962, MK852171, and MK852172) revealed close similarities (99 to 100%) with other reported sequences. Two branches of the tree had high bootstrap values. In the first branch, MK840962, MK852171, and MK852172 clustered with OvHV-2 sequences isolated from India (MF685297), Japan (LC203437), Egypt (KP015737), Turkey (JN084009), Italy (KJ420947), and Brazil (KJ658293). A second complex branch showed relationships between the sequences obtained from the Rawalpindi and Islamabad districts and those of isolates from Brazil (HQ223415), India (MK059980 and KR092147), and South Africa (EU851178); those relationships had high bootstrap values (**Fig. 2B**), but the connections were more distant than those in the first branch of the phylogenetic tree.
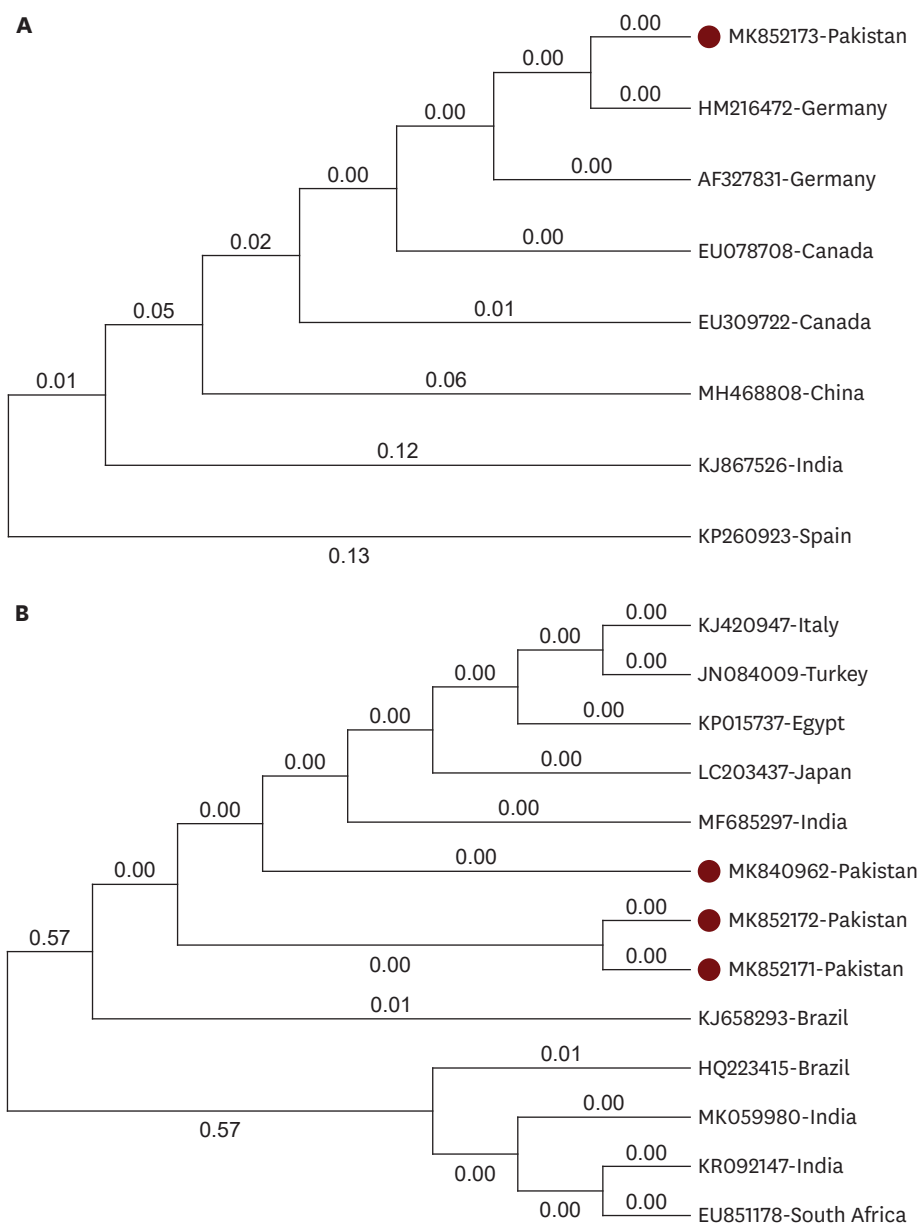
Fig. 2. Phylogenetic analysis of the ovine herpesvirus-2 POL (A) and ORF75 (B) tegument protein gene segment sequence, and closely related reference sequences obtained from GenBank; constructed using the maximum likelihood method in MEGA 7 software.

DISCUSSION

In 1981, a reported case of MCF in a buffalo heifer in a village near Faisalabad, Pakistan, was based on clinical signs and symptoms [17]. The animal showed all of the characteristic signs and symptoms of MCF and died after an illness of 19 days. No post-mortem examination of the carcass was performed [17]. In neighboring countries like India [14,23], China [22], and Iran [15,16], reports of MCF and the prevalence of OvHV-2 have been published from time to time. However, since the 1981 report by Ashfaque and Mohammad [17], no reports on MCF cases in Pakistan have been published. The present study is, therefore, the first PCR-confirmed report of the prevalence of OvHV-2 in Pakistan.

It is well-established that sheep act as asymptomatic carriers of OvHV-2, and in Pakistan mixed farming of sheep, goats, cattle, and buffalo is common practice, and the various species are housed in close proximity to each other. Moreover, the animals often share common feeding areas [8,17]. In the present study, heminested PCR was used to detect the presence of OvHV-2 DNA in blood samples of apparently healthy animals. The heminested PCR method used to detect OvHV-2 DNA is highly sensitive and specific, and it is the OIE-recommended diagnostic tool for identifying sheep-associated MCF [9,24]. DNA sequence analysis confirmed the similarity of our sequences with other OvHV-2 ORF75 tegument protein and POL gene sequences reported globally. Our results revealed an overall prevalence of OvHV-2 of 40% in the Rawalpindi and Islamabad districts. Of the samples taken from apparently healthy animals, 48% (sheep), 52% (goats), 39% (cattle), and 26% (buffalo) were found to be OvHV-2-positive, based on the heminested PCR results (**Table 1**).

Previous international studies into the prevalence of OvHV-2 have indicated that goats, believed to be natural hosts of caprine herpesvirus 2 (CpHV-2), are also susceptible to infection by OvHV-2 [15,23-28]. Studies on wild ruminants in Iran indicated a 10% OvHV-2 prevalence in goats [15]. The prevalence of OvHV-2 among the goats in our study area was higher; however, it was consistent with that reported for goats sampled in the Kashmir valley (61%), a region where mixed farming and grazing of goats and sheep is practiced [24,27]. While goats have been shown to transmit CpHV-2 to susceptible species [5,28], it has not yet been demonstrated that goats can transmit OvHV-2 to naive animals [29]. Susceptible species are considered dead-end hosts as they do not transmit the infection to other susceptible species or host species (sheep and goat). The lack of infectivity is likely because the virus remains cell-associated in these species; thus, cell-free viruses are not produced. Though the majority of cases of OvHV-2 infection in goats appears to be subclinical, reports have associated OvHV-2 infection in goats with various clinical symptoms, including corneal opacity and pyrexia with neurological signs [30,31]. Further research is required to elucidate the effect of OvHV-2 on the health and productivity of goats and determine whether goats can act as a reservoir animal and be a source of OvHV-2 infection in susceptible animals.

The results of our study also indicated high OvHV-2-positivity levels in samples taken from apparently healthy cattle (40%) and buffalo (26%). This may suggest that, under natural exposure conditions, subclinical OvHV-2 infections regularly occur in cattle and buffalo. This observation is consistent with experimental studies demonstrating that OvHV-2 infection can occur in cattle without concurrent development of clinical MCF [32,33]. Furthermore, experimental evidence has indicated that whether an animal develops clinical signs of MCF depends on the infectious dose of OvHV-2 that the animal receives [34].

Several studies in Pakistan have focused on diseases in cattle and buffalo, including rinderpest, peste des petits ruminants (PPR), foot and mouth disease (FMD), theileriosis, and babesiosis [17,35], which share clinical symptoms with MCF. Those authors have reported large percentages of symptomatic animals tested negative for the presence of the pathogen(s) under investigation. MCF is not tested in Pakistan routinely; thus, its effect on the national herd and economy may be underestimated. Movements of animals between cities for trade purposes and during religious and social festivals result in the mixing of different animal species, providing opportunities for transmission of infections such as MCF. In Pakistan and neighboring countries, the free movement of goats and sheep across national borders makes MCF a transboundary disease [14]. Phylogenetic analysis has shown that the OvHV-2 detected in Pakistan is not markedly separated from other globally identified

isolates. Diagnostic testing, using methods such as nested PCR, should therefore be used to identify the pathogen in all suspected cases of MCF, whether the case occurs within the national herd or is a transboundary case. Furthermore, improved laboratory diagnostic capability through increased availability of sensitive and specific molecular tools like PCR, which enables detection of viral DNA, can provide a reliable test to distinguish MCF cases from other diseases producing similar clinical symptoms in the affected species.

MCF causes significant economic losses worldwide in major ruminant species. Moreover, it poses a threat to other susceptible species that are housed in close proximity to the infected species [14]. While transmission of OvHV-2 over distances of 5 km between lambs and bison without physical contact has been reported [8,36], the close proximity of susceptible species with infected sheep, and potentially goats, on mixed pastures increases the risk of virus transmission and MCF development. Since no vaccines for MCF are currently available, control measures such as segregating animals by a 5 km distance or housing lambs separate from susceptible species may reduce MCF incidence [14,37].

As the geographical spread of MCF is unknown in Pakistan, implementation of a differential diagnostic testing approach by veterinarians and government officials could lead to improved husbandry/planning during potential outbreaks. Investigation of the epidemiology and pathophysiology of OvHV-2, particularly as they relate to cellular tropism, virus replication, and viral protein expression, may help in characterizing the virus and its association with MCF in cattle and buffalo. Development of pen-side testing for MCF and other related diseases with similar clinical symptoms (like FMD and PPR) could help in the early diagnosis of a disease outbreak.

MCF is a fatal lymphoproliferative disease of susceptible ruminants and causes substantial economic losses among livestock farmers worldwide. Our study demonstrated the prevalence of OvHV-2 in Pakistan in several carrier species as well as in apparently healthy susceptible species (cattle and buffalo). To the best of our knowledge, this is the first report on the prevalence of OvHV-2 in Pakistan. A large-scale detailed study is required to better understand the prevalence and pathogenesis of OvHV-2 in natural and susceptible hosts. Factors affecting the virulence of OvHV-2 in susceptible species should also be investigated as the present study revealed OvHV-2 presence in apparently healthy susceptible animals.

ACKNOWLEDGMENTS

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