First Study of Molecular detection of Ovine Herpesvirus 2 DNA using heminested PCR in Pakistan

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Research

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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). AlHV-1 is known to cause wildebeest-associated MCF whereas OvHV-2 is known to cause sheep-associated MCF. AlHV-1 and OvHV-2 cause inapparent infection in their reservoir hosts (wildebeest for AlHV-1 and sheep for OvHV-2) but fatal lymphoproliferative disease in susceptible species.

Methods

This study was aimed to find prevalence of OvHV-2 in sheep, goat, cattle and buffalo in Rawalpindi and Islamabad cities of Pakistan, using molecular and phylogenetic methods. Blood samples were collected consisting of sheep (n = 54), goat (n = 50), cattle (n = 46) and buffaloes (n = 50) from slaughter houses and farms. The samples were subjected to hemi-nested PCR, sequencing, and phylogenetic analysis of OvHV-2 POL gene and OvHV-2 tegument protein gene.

Results

The highest percentage of positive samples was found to be in sheep (13%) whereas positive samples from goat, cattle and buffaloes had percentage of 11%, 9% and 6.5% respectively. Four positive PCR products from sheep samples were also sequenced. The sequences obtained were submitted to NCBI GenBank database under the accession numbers of MK852173 for POL gene, and MK840962, MK852171 and MK852172 for ORF75 tegument protein gene. Phylogenetic analysis revealed close identities with some sequences of this virus from worldwide including neighbouring countries including China, India and Iran.

Conclusion

This study is the first study in Pakistan on the prevalence of OvHV-2 in carrier and susceptible species. This report provides important data about the presence of OvHV-2 in apparently healthy cattle and buffaloes, which might transmitted to them from OvHV-2 positive sheep or goats. It is concluded that a countrywide, large scale OvHV-2 prevalence study should be conducted to avoid economic losses due to any outbreak of MCF.

Introduction

The Pakistani livestock sector contributes approximately 11% to Pakistans's annual gross domestic product and provides employment to approximately 62% of the population in rural areas when subsidiary industries are taken into account (1). The national herd of Pakistan incorporates 29.6 million cattle, 27.3 million buffalo, 53.8 million goats and 26.5 million sheep (Habib et al., 2016). Infectious diseases that affect animal that constitute the national herd impact both the security of the food chain and the wider economy.

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, 3, 4, 5). MCF may be caused by any of the gamma herpesviruses, identified so far, that comprise the macavirus classification group. The two most well studied of these macaviruses, Ovine Herpesvirus-2 (OvHV-2) and Alcelaphine Herpesvirus-1 (AlHV-1), 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 the development of MCF. Though, OvHV-2 transmission to susceptible animals, via aerosol, has been reported to occur up to 5 km from where reservoir animals are located (8, 9) the most common route of transmission is most likely direct contact of susceptible species with nasal secretions of reservoir animals, containing infectious virus, on mixed grazing areas.

Clinically, MCF is characterized by fever, excessive salivation, nasal and ocular discharge. Lesions are usually present on buccal cavity and muzzle. Enlarged lymph nodes is also a characteristic sign of MCF. Post mortem lesions include presence of petechial haemorrhages on the mucosa of buccal cavity, in the gastrointestinal and respiratory tracts (9, 10). Symptoms of MCF in susceptible
species are similar to those of other diseases, such as vesicular stomatitis virus, foot and mouth disease and rinderpest (6, 12). Clinically affected susceptible hosts do not shed infectious virus and are considered as dead end hosts (13, 14).

MCF cases have been documented in both India (15) and Iran (16, 17), which border Pakistan. In Pakistan in most rural areas, livestock farms, animal hospitals and research centres, it is a common practice to keep MCF reservoir and susceptible animals in close proximity (18, 19). It is likely, therefore that the lack of documented cases of MCF in Pakistan, is a result of underreporting due to a lack of testing and awareness amongst veterinarians. In this study, for the first time in Pakistan, PCR testing of blood samples derived from abattoirs and farms was used to investigate the prevalence of OvHV-2 in cattle, buffalo, sheep and goats in Rawalpindi District and Islamabad city.

Materials And Methods

Samples collection

Two hundred blood samples consisting of sheep (n = 54), goat (n = 50), cattle (n = 46) and buffaloes (n = 50) samples were randomly collected from slaughter house (Sihala slaughter house, Rawalpindi) and farms of Rawalpindi and Islamabad, Pakistan. Blood samples were collected in ethylene diamine tetra acetic acid (EDTA) containing tubes and transported to the Virology laboratory of Department of Parasitology and Microbiology, FV&AS, Pir Mehr Ali Shah, Arid Agriculture University, Rawalpindi for further processing. On arrival at the labs the blood samples were refrigerated at 4°C.

Processing of Samples and DNA isolation

The buffy coat layer was removed carefully for DNA extraction and stored in separate tubes at -20°C. DNA isolation was performed using 100–120 µl from the buffy coat layer was performed using the PureLink® Genomic DNA Kit (Invitrogen). Concentration of extracted DNA in the samples was measured using a Nano-Drop spectrophotometer. DNA samples were stored at -20°C.

Construction Of The OvHV-2 Positive Control

A section of the polymerase gene was amplified from DNA, extracted from BJ1035 cells (20), using Qiagen's DNA blood and tissue kit (Qiagen, UK). Regions of the polymerase gene (POL gene) were amplified in accordance with the primers specified by (21). The amplified product was purified, using Qiagen's PCR purification kit (Qiagen,UK) in accordance, and cloned into Topo vector pcr2.1+ (Life Technologies, UK). The purified DNA was used as a positive control in all documented OvHV-2 POL gene PCRs.

Heminested PCR to amplify OvHV-2 POL and tegument gene

Heminested PCRs were performed to amplify fragments of OvHV-2 POL gene using the extracted DNA samples. The following primer sets were used (22)

Primer POL1: 5’-GGC(CT)CA(CT)AA(CT)CTATG CTACTC CAC-3’
Primer POL2: 5’-ATT(AG)TCCACAAACTGTTTTGT-3’
Primer OHVPOL: 5’-AAAAACTCAGGGCCATTCTG-3’

DNA samples along with a positive and a negative control were selected for PCR. Primer POL1 and Primer POL2 were used in the first step for 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 minutes; followed by 25 cycles of 94 °C for 60 seconds, 60 °C for 60 seconds and 72 °C for 60 seconds; with a final extension at 72 °C for 10 minutes. For negative control nuclease free water was used. For Secondary amplification primer OHVPOL and POL2 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 was run on a 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 following primer sets were used (21).
Primer 556: 5’-AGTCTGGGTATATGAATCCAGATGGCTCTC-3’
Primer 555: 5’TTCAGGAGTAGTGGCGAGCGAAGGCTTC-3’
Primer 755: 5’-AAGATAAGCACCAGTTATGCATCTGATAAA-3’

The primer 556 and 755 amplifying fragment of 422 bp for the primary amplification step of the PCR. Primer 556 and Primer 555 were used in the secondary amplification step to amplify a fragment of 238 bp product. In both amplifications steps the PCR and electrophoresis conditions were same as that of used for heminested PCR for POL gene.

**Sequence and Phylogenetic Analysis**

Four positive PCR products from DNA samples taken from different sheep, one from POL gene PCR and three from tegument protein gene PCRs from the second amplification step were sent for gene sequencing to Macrogen® Korea. Primer OHVPOL and POL2 were used to sequence POL gene PCR product and primer 556 and 555 were used to sequence tegument protein gene PCR product. Sequences derived from this study and those obtained from the GenBank database were aligned by the CLUSTAL_W method 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 with neighbor joining to construct the phylogenetic tree (19). The statistical significance of the relationships obtained was determined by bootstrap re-sampling analysis with 1000 repetitions.

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

**Results**

Two hundred blood samples were taken randomly from different species included 46 cattle, 50 buffalo, 50 goats and 54 sheep from different farms, small holdings at household level and slaughter house of Rawalpindi and Islamabad Pakistan. All the animals were apparently healthy at the time of sampling. DNA was isolated from buffy coat of each of the blood sample and the extracted DNA was subjected to heminested PCR, using primers recommended by the OIE for the detection of OvHV-2. Initial screens of the samples were carried out using primers targeted towards the virus POL gene. A plasmid containing the region of the OvHV-2 POL gene, targeted by the POL primers, derived from the OvHV-2 immortalised cell line BJ1035 was utilised as a positive control. Samples registered as positive for OvHV-2 had 172 bp amplicon and no amplicon was observed in negative control samples tested at the same time (Fig. 1a). The positive DNA samples were further PCR tested for amplification of a region of tegument protein gene (Fig. 1b). Out of 200 samples, 79 were determined to be positive for both OvHV-2 POL and tegument genes using hemi-nested PCR (Table 1). The highest percentage of positive samples was found to be in sheep (13%) whereas positive samples from goat, cattle and buffaloes had percentage of 11%, 9% and 6.5% respectively (Table 1).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Species</th>
<th>No. of Samples</th>
<th>Positive Samples</th>
<th>% out of Total</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Cattle</td>
<td>46</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>Buffalo</td>
<td>50</td>
<td>13</td>
<td>6.5</td>
</tr>
<tr>
<td>3</td>
<td>Goat</td>
<td>50</td>
<td>22</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>Sheep</td>
<td>54</td>
<td>26</td>
<td>13</td>
</tr>
</tbody>
</table>

**Molecular phylogenetic analysis of regions of POL gene and tegument protein gene of OvHV-2**

The nucleotide sequences of the POL gene region and tegument protein gene region of four OvHV-2 positive samples were compared with seven sequences and ten sequences respectively, reported by other researchers from around the world. Phylogenetic trees were
transmission of infections like MCF. In Pakistan and neighbouring countries free movement of goats and sheep across the borders make for trade purposes during religious and social festivals caused mixing of different animal species which provides opportunity for MCF routinely and so its impact on the national herd and economy may be underestimated. Movements of animals between cities symptomatic animals that tested negative for the presence of the pathogens under investigation. Veterinarians in Pakistan do not test for MCF or other viral infections in apparently healthy animals. Most experimental studies following OvHV-2 infection have focused on cattle and buffaloes as a reservoir animal, and source of infection for susceptible animals for OvHV-2. It is a well-established fact that sheep act as asymptomatic carriers of OvHV-2 and in Pakistan it is a common practice to keep sheep, goats, cattle and buffaloes in close proximities to each other, often with shared common feeding areas. In the present study, heminested PCR assays was used to find the presence of OvHV-2 DNA in blood of apparently healthy animals. The hemi-nested PCR used to detect OvHV-2 DNA is highly sensitive, specific and is the diagnostic tool for SA-MCF recommended by the OIE. DNA Sequence analysis confirmed the similarity of our Sequences with other OvHV-2 sequences isolated from India (MF685297), Japan (LC203437), Egypt (KP015737), Turkey (JN084009), Italy (KJ420947) and Brazil (KJ658293). A second complex branch with isolates from Brazil (HQ223415) India (MK059980 and KR092147) and South Africa (EU851178) were found (Fig. 2b), with high bootstrap value, to be more distant from the sequences obtained from the Rawalpindi and Islamabad study area.

Discussion

In 1981 a case of MCF was reported on the basis of clinical signs and symptoms (see Additional File 1) (18). The disease was reported in a buffalo heifer in a village near Faisalabad. The animal showed all the signs and symptoms of MCF and died after an illness of 19 days. No postmortem examination of the carcass was performed (18). In neighbouring countries like India (15, 24), China (23) and Iran (16, 17) documented reports of MCF and the prevalence of OvHV-2, have been published from time to time. However, since the report from Ashfaqe and Mohammad in 1981, no reports on cases of MCF in Pakistan have been published. This present study is therefore the first confirmed report of the prevalence of OvHV-2 amongst the national herd of Pakistan.

It is a well-established fact that sheep act as asymptomatic carriers of OvHV-2 and in Pakistan it is a common practice to keep sheep, goats, cattle and buffaloes in close proximities to each other, often with shared common feeding areas. In the present study, heminested PCR assays was used to find the presence of OvHV-2 DNA in blood of apparently healthy animals. The hemi-nested PCR used to detect OvHV-2 DNA is highly sensitive, specific and is the diagnostic tool for SA-MCF recommended by the OIE (9, 25). DNA Sequence analysis confirmed the similarity of our Sequences with other OvHV-2 sequences isolated from India (MF685297), Japan (LC203437), Egypt (KP015737), Turkey (JN084009), Italy (KJ420947) and Brazil (KJ658293). A second complex branch with isolates from Brazil (HQ223415) India (MK059980 and KR092147) and South Africa (EU851178) were found (Fig. 2b), with high bootstrap value, to be more distant from the sequences obtained from the Rawalpindi and Islamabad study area. Of the samples taken from apparently healthy animals, 48% of the sheep, 52% of the goats, 39% of the cattle, and 26% of the buffalo tested were found to be positive for OvHV-2 using PCR (Table 1).

Previous studies into the prevalence of OvHV-2 from around the world have indicated that goats, believed to be the natural hosts of Caprine Herpesvirus 2 (CpHV-2), are also susceptible to infection by OvHV-2 (16, 24–29). Studies on wild ruminants in Iran, indicated an OvHV-2 prevalence in goats of 10% (16). The prevalence of OvHV-2 amongst the goats in our study area is higher, however it is consistent with that reported by sampling goats tested in the Kashmir valley (61%) where mixed grazing of goats and sheep is practiced (25, 28). Whilst, goats have been shown to transmit CpHV-2, to susceptible species (5, 29) it has not yet been demonstrated that goats can transmit OvHV-2 to naïve animals (30). Though the majority of cases of OvHV-2 infection of goats appear to be subclinical, reports have tied OvHV-2 infection with clinical symptoms in goats including corneal opacity and pyrexia with neurological signs (31, 32). Further research is required to understand both the impact of OvHV-2 on the health and productivity of goats and whether goats can act as a reservoir animal, and source of infection for susceptible animals for OvHV-2.

Results of our study also indicated high levels of samples taken from apparent healthy cattle (40%) and buffalos (26%) to be positive for OvHV-2. This may suggest that under natural exposure conditions sub-clinical OvHV-2 infections regularly occur in cattle and buffaloes. This observation is consistent with experimental studies, using OvHV-2, that have demonstrated infection can occur in cattle without concurrent development of clinical MCF (33, 34). Furthermore, experimental evidence has indicated that whether an animal develops clinical signs of MCF may depend on the infectious dose of OvHV-2 that the animal receives (35).

Several studies in Pakistan focused on diseases in cattle and buffaloes such as Rinderpest, peste des petits ruminants (PPR), foot and mouth disease (FMD), theileriosis and babesiosis (18, 36), which share clinical symptoms with MCF, have reported large percentages of symptomatic animals that tested negative for the presence of the pathogens under investigation. Veterinarians in Pakistan do not test for MCF routinely and so its impact on the national herd and economy may be underestimated. Movements of animals between cities for trade purposes during religious and social festivals caused mixing of different animal species which provides opportunity for transmission of infections like MCF. In Pakistan and neighbouring countries free movement of goats and sheep across the borders make...
MCF a transboundary disease (15). Phylogenetic analysis has shown that the OvHV-2 detected in Pakistan, showed no geographic separation from isolates from around the world. Diagnostic tests, using nested PCR, should therefore be able to identify all cases of MCF, caused by OvHV-2 whether they have resulted from within the national herd or from transboundary cases. Furthermore, the development of improved laboratory diagnostic capability through availability of sensitive and specific molecular tools like PCR, which enables detection of viral DNA, provides a reliable test to distinguish MCF cases from other diseases that induce similar clinical symptoms in affected species.

MCF causes significant economic losses worldwide in major ruminant species. MCF also poses threat to other susceptible species which are housed in close proximity to infected species (15). Whilst transmission of OvHV-2 over distances of five kilometres between lambs and bison without any physical contact has been reported (8, 37), close proximity of susceptible species with infected sheep, and potentially goats, on mixed pasture provides a greater risk of transmission of virus and development of MCF. Since there are no vaccines available for the disease so far and on control measures is the best strategy (15), such as segregating animals by 5 km or housing lambs separate from susceptible species, to reduce the incidence (38).

Continued investigation of the epidemiology and patho-physiology of OvHV-2, particularly in regards to cellular tropism, virus replication, and viral protein expression, is necessary for a more complete understanding of the virus and its association with MCF in cattle and buffaloes. Development of penside testing for MCF and other related with similar clinical symptoms diseases (like FMD and PPR) could help in the early diagnosis of the diseases and lead to better husbandry/ planning during potential outbreaks for veterinarians and government officials.

**Conclusion**

MCF is a fetal lymphoproliferative disease of susceptible ruminants and causes enormous economic losses to livestock farmers worldwide. In Pakistan the research on MCF is negligible. Our study demonstrated the prevalence of OvHV-2 in carrier species as well as in apparently healthy susceptible species (cattle and buffaloes). Present study is the first report to the best of our knowledge, on the prevalence of OvHV-2 in Pakistan. A large scale detailed study is required to achieve a better understanding of prevalence and pathogenesis of the virus in natural and susceptible hosts. Factors affecting the virulence of OvHV-2 in susceptible species are also needed to be investigated as the present study has shown the presence of virus in apparently healthy susceptible animals.

**Abbreviations**

MCF
Malignant catarrhal fever
AlHV-1
Alcelaphine herpesvirus 1
OvHV-2
Ovine herpesvirus 2
WA-MCF
Wildebeest-associated MCF
SA-MCF
Sheep-associated MCF
POL gene
Polymerase gene
Tegument protein gene
ORF75

**Declarations**

**Ethics Approval**

All the sampling was done under strict ethical conditions, with the permission of the owners. The animals were handled and samples were taken by Pakistan Veterinary Medical council certified professional veterinary clinicians/practitioners (Doctor of Veterinary Medicine). This work was approved by Ethics Committee for use of Animal & Human Subjects, Arid Agriculture University and Higher
education commission of Pakistan (SRGP-620). All the methods used including check up and taking samples were done without giving any harm to the animals. Also no anaesthesia, euthanasia, or any kind of animal sacrifice was the part of the study.

Consent to publication

Not Applicable

Availability of data and material

Not Applicable

Competing interests statement:

The authors declare that they have no financial and non-financial competing interests.

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Authors’ contributions

AR, ID and RGD Conceived and designed the experiments. AR, ID, NA performed the experiments, AR, ID, SUR, MAAS, AY, RMB Contributed reagents/materials/analysis tools. AR and ID were the major contributor in writing the manuscript. All authors read and approved the final manuscript.

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References

10. OIE


Figures

Figure 1

a: Nested PCR results showing approximately 172bp bands of POL gene. The primers POL1 and POL2 target a segment of the DNA polymerase gene which is conserved in both OvHV-2 and AlHV-1, amplifying a fragment of 386bp. OHVPol is specific primer for OvHV-2 which amplify 172bp products. L1 and L2: Samples showing positive results, P: Positive control, M: 100bp Ladder, N: Negative control. b: Nested PCR results showing approximately 238bp bands of tegument protein gene. L1, L4-L7: Samples showing positive results. M: 100bp Ladder, N: Negative control.

Figure 2
a: The phylogenetic analysis of the segment of POL gene sequence and their closely related reference sequence obtained from GenBank constructed using Maximum Likelihood method in MEGA 7 software. b: The phylogenetic analysis of the segment of tegument protein gene sequence and their closely related reference sequence obtained from GenBank constructed using maximum likelihood method in MEGA 7 software.

**Supplementary Files**

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- supplement1.png