**Emerging Evidence of Border Disease Virus in Indian Buffaloes co infected with Sheep Associated MCF: Clinical Signs, Molecular Characterization, and Phylogenetic Insights**

**ABSTRACT:**

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| **Aim:** To investigate the etiological agents involved in mortality of buffaloes in Andhra Pradesh, India.  **Study design:** This was an observational diagnostic study involving clinical evaluation, molecular diagnostics, sequencing and phylogenetic analysis to confirm the etiological agent.  **Place and Duration of Study:** The study was conducted in Srungavarapukota, Vizianagaram,  Andhra Pradesh, India during November 2023.  **Methodology:** Three adult buffaloes aged between 5 to 6 years showing clinical signs such as pyrexia, congested eyes with ocular discharge, respiratory distress, mucopurulent nasal discharge and diarrhoea were suspected for viral infections such as Infectious Bovine Rhinotracheitis (IBR), Malignant Catarrhal Fever (MCF), or Bovine Viral Diarrhoea (BVD). To identify the etiological agents involved blood and Nasal swabs were collected aseptically and molecular diagnostic assays such as PCR were employed to detect IBR by amplifying the glycoprotein B (gB) gene, MCF by Ovine herpesvirus 2 (OvHV-2) tegument gene and BVD by Pestivirus-specific 5′ UTR. Additionally, samples were tested using the BVD P80 antigen capture ELISA. PCR products from one animal that tested positive for both OvHV-2 and Pestivirus were sequenced and Phylogenetic trees were constructed using the resulted sequences.  **Results:** All three buffaloes tested negative for IBR by PCR and for BVD by ELISA. However PCR testing revealed that all samples were positive for both OvHV-2 and Pestivirus. In BLAST analysis the PCR products were confirmed as OvHV-2 and Border Disease Virus (BDV). Phylogenetic analysis revealed that the OvHV-2 sequence clustered with previously reported isolates from Andhra Pradesh, while the pestivirus sequence showed high genetic identity with a BDV-3 isolate reported in sheep from NIHSAD, Bhopal, India and exhibited significant genetic divergence from Classical Swine Fever Virus (CSFV), Bovine Viral Diarrhea Virus (BVDV), and other BDV subgenotypes.  **Conclusion:** The findings of this study conclusively demonstrate that the affected buffaloes were co-infected with sheep associated Malignant Catarrhal Fever Virus (OvHV-2) and Border Disease Virus. The synergistic action of these viruses exacerbated the pathogenicity resulting in mortality of the three affected animals. Notably, sheep acted as primary reservoir for both diseases, underscoring the risk of interspecies transmission within mixed farming systems. |

*Key words: Buffaloes, Border Disease, Malignant catarrhal fever, Polymerase Chain reaction, Phylogentic analysis.*

1. **INTRODUCTION:**

**The term “Co infection”** refers to “simultaneous infection of host by different pathogens, mainly viruses”. While viral diseases are generally associated with a single causative agent, in natural settings, animals frequently harbor multiple infectious agents concurrently, with disease outcomes influenced by the combined effects of these pathogens (Kumar, et al., 2018). For instance, **Bovine Viral Diarrhea (BVD)** and **Malignant Catarrhal Fever (MCF)** are viral diseases that can infect artiodactyls such as cattle, pigs, sheep, goats and deer causing ocular, respiratory, and gastrointestinal symptoms.

Bovine Viral Diarrhea (BVD) is caused by Bovine Viral Diarrhea virus (BVDV) which belongs to family Flaviviridae, genus Pestivirus along with Border disease virus (BDV) and Classical Swine Fever virus (CSFV). These three viruses were traditionally named after the affected species. However, all three are capable of crossing species barriers to infect a wide range of hosts within the Artiodactyla (Becher, et al., 1997). Although Border disease virus is primarily recognized as reproductive disease of sheep and goats, it can also infect cattle and pigs.Sheep act as carriers and can cause symptoms such as abortions, fetal mummification and diarrhea in cattle leading to economic losses similar to those caused by Bovine viral diarrhea (Cranwell, et al., 2007).

All Pestiviruses are single standard positive sense RNA viruses with a genome length of 12.3 kb length, coding for four structural proteins and seven non structural proteins within a single open reading frame flanked by5’ and 3’ untranslated regions (UTR).The 5’- UTR is highly conserved among the pestiviruses and can be efficiently amplified by RT\_PCR from field samples. Consequently, this region has been used widely for the development of nucleic acid based diagnostics, commonly referred as pan pestivirus PCR. Although various PCR protocols exist for the detection of BDV, BVDV, and CSF, pan-pestivirus PCR exhibits high sensitivity across different pestiviruses and is most frequently employed (Braun, et al., 2024). Genotyping of pestiviruses in positive samples is subsequently achieved through nucleotide sequencing (Ridpath, 2003). Peletto et al. (2016) demonstrated that a commonly used BDV-specific nested PCR failed to amplify a novel BDV genotype (BDV-8), which was only detected using pan-pestivirus primers such as the 5’-UTR. This finding underscores the necessity for broader molecular approaches and phylogenetic analysis to ensure accurate BDV detection and genotyping.

Malignant catarrhal fever (MCF) is an acute, fatal and sporadic disease caused by two types of Gamma herpes viruses namely Alcelaphine herpes virus 1 (AIHV-1) and Ovine herpes virus 2 (OvHV-2) which affect all artiodactyl ruminants. OvHV-2 is commonly reported in cattle and buffaloes, transmitted from sheep where mixed farming is practiced (Sood, et al., 2014). The detection of viral DNA is recognized as one of the confirmatory test for MCF by the WOAH Terrestrial Manual 2018.

Laboratory diagnosis of a disease typically involves correlating clinical signs to a specific pathogen to confirm its causative role. However, in practice, multiple pathogens may contribute to the disease process. Consequently, understanding the clinical impact, accurate diagnosis, and effective treatment and control of such viral infections is of paramount importance.

1. **MATERIALS AND METHODS:**

**2.1 Case history:** Three adult buffaloes, approximately 5 to 6 years age from Srungavarapukota mandal in Vizianagaram district of Andhra Pradesh, India, were presented with symptoms including persistent high-grade fever (pyrexia ranging from 103°F to 105°F), congested eyes with ocular discharges, respiratory distress, mucopurulent nasal discharges. Additionally, all three animals showed signs of gastrointestinal involvement, particularly watery diarrhea. Despite supportive care, all three animals succumbed to the disease within one week of the onset of symptoms. Unfortunately, necropsy (post-mortem) examinations could not be conducted due to local constraints and rapid disease progression.

To identify potential etiological agents, ante mortem clinical specimens including EDTA blood and nasal swabs were aseptically collected from each animal during the symptomatic phase. Giventhe symptoms indicative of BVD, MCFand IBR, Polymerase Chain Reaction (PCR) for all three diseases and ELISA for BVD were employed to screen the samples.

**2.2 Detection of BVDV by ELISA:-**Samples from three animals were tested for BVDV with commercially available BVD P80 Antigen capture ELISA kit (ID screen) which detects the BVDV/BDV P80 (NSP2-3) protein in the samples such as serum, plasma, tissues and nasal Swabs from cattle, Sheep and goat.

**2.3 Detection of viruses by PCR and RT-PCR:** DNA was extracted by using DNeasy blood and tissue kit (Qiagen) and total RNA was isolated by using TRIzol (Invitrogen, Thermo Fisher).Thedetails of PCRs employed are detailed in the table 1. As part of post-PCR analysis, the PCR products were electrophoresed on 1.5% agarose gel in 1X TBE buffer with 0.5 μg/ml of ethidium bromide and visualized under UV illumination.

**Table1. Details of PCR’s employed for testing samples**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Disease** | **Target Gene** | **Type of PCR** | **Volume of Reaction Mix** | **Annealing temperature** | **Product size (bp)** | **Reference** |
| IBR | UL27 (gB) | Conventional PCR | Emerald Amp HS PCR Master Mix (Takara bio) - 12.5 µl, Primer pair (20pm/µl)-1 µl each, template- 5 µl and Nuclease free water- to make up to 25 µl | 58◦C, 30 sec | 484 | Kumar, et al., 2020 |
| MCF | Tegument gene of OvHV-2 | Hemi Nested PCR | 63◦C, 30 sec | 422 and 238 | Sood, et al., 2014 |
| Pestivirus | 5’UTR | RTPCR | Primescript one step RT PCR MM- 25 µl, F&R primers- 1µl, Taq enzyme- µl, Template-1 µl, NFW make up to 50 µl | 58◦C, 15 sec | 288 | Vilcek, et  al., 2001 |

**2.4 Sequencing and Phylogenetic analysis:** For virus characterization, amplified product from one animal from both pestivirus PCR and MCF outer PCR were sequenced using Sanger sequencing. Chromatograms were analyzed with Chromas software version 2.6.6, and sequence errors were corrected. The contig sequence was aligned using Codon code aligner and subjected to NCBI nucleotide blast for virus confirmation. Phylogenetic analysis for BDV was performed by aligning reference sequences of various BDV subgenotypes 1-7, BVDV-1, BVDV-2 (Mishra, et al., 2016) and Andhra Pradesh CSFV using the Clustal W program in MEGA 11 software. The phylogenetic tree was constructed using Neighbour-joining method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the BDV isolate (VBRI, AP). Similarly phylogenetic analysis for MCF was also constructed as stated above by aligning reference sequences of various MCF isolates selected from Andhra Pradesh, India and worldwide.

3. results and discussion

**3.1 Results:**

**3.1.1 ELISA:** In the BVD P80 Antigen capture ELISA (ID screen) all three animals tested negative for BVDV/BDV.

**3.1.2 PCR & Molecular analysis: -**

PCR analysis revealed that all three animals tested positive for both Pestivirus and OvHV-2 (Images 1 & 2), while they were negative for IBRV. Sequence analysis of the Pestivirus, conducted using NCBI Blast, identified it as Border Disease Virus, demonstrating a 98% identity with Accession No. ON227057 from the Institute of High Security Animal Diseases, Anand Nagar, Bhopal, Madhya Pradesh 462022, India (Kalaiyarasu, et al., 2024). In the phylogenetic tree (Figure 1), the current isolate was positioned within the BDV-3 clade, exhibiting significant genetic divergence from other BDV subgenotypes and other pestivirus species such as BVDV-1, BVDV-2, and CSFV. The complete genetic sequence is accessible for reference and further investigation at NCBI with Accession No. PQ862858.1.

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Image 1: Hemi-nested PCR for OvHV-2 targeting tegument gene with product sizes of 422 bp and 238 bp

Image 2: RT PCR for Pestivirus targeting 5’-UTR gene with product size of 288 bp

UTR with

J04358.2: CSFV,Tuebingen, Germany

OR771719.1: CSFV, Andhra Pradesh, India

NC\_076029.1: BVDV-1,Bethesda,USA

FM163382.1 BDV-7, via Salvemini, 1, Italy

**PQ862858.1: BD/VZM/1/VBRI/Andhra Pradesh, India \*\*\*\***

ON227057.1:1-241 BDV-3, Bhopal, MP, India

EF693984.1 BDV 5,Antipolis, France

AF144618.2:BDV2,Giessen D, Germany

GQ902940.1:BDV3,Kalvehave,Denmark

NC\_039237.1:BVD-2, Ames, USA

DQ275625.1:BDV 4, Surrey, UK

EF693990.1:BDV6,Antipolis, France

AF037405.1 BDV 1, Giessen 35392, Germany

95

65

60

78

44

45

40

29

28

7

Fig 1: Phylogenetic tree based on 5’-UTR nucleotide sequence ofpestivirus. Evolutionary analysis was conducted in MEGA 11 Neighbour-joining method with bootstrap consensus tree inferred from 1000 repetitions. The sample VBRI AP in our study is marked with an asterisk.

Sequence analysis of the 422 bp PCR product of MCFconducted using NCBI Blast, confirmed it as MCF demonstrating a 99% identity with IndianMCF isolates, specifically those with accession numbers MF977714.1 from SVVU, Andhra Pradesh; PQ862864.1 from VBRI, Andhra Pradesh; and OR604336 from NIVEDI, Bengaluru. In the phylogenetic analysis (Figure 2), the current isolate was grouped with isolates from Andhra Pradesh and Karnataka, a neighboring state, and branched separately from those of countries outside India. The MCF isolate from the present study has been submitted to NCBI with accession number PQ862859.1.

NC 007646.1:121102-121528 OvHV2 strain, BJ1035, USA

KR092147.1:1-422 OvHV2 strainNIHSAD, Bhopal, India

ON375583.1:2-417 OvHV2 isolate, Mexico

KC123170.1:1-421 OvHV2 isolate, Brazil

PQ862864.1:1-403 OvHV2 isolate, VBRI,Andhra Pradesh, India

MF977714.1:1-423 OvHV2 isolate, SVVU, Tirupathi, Andhra Pradesh

**PQ862859.1:1-427 OvHV2 isolate, MCF/VZM/1/VBRI/Andhra Pradesh, India\*\*\***

OR604336.1:1-421 OvHV2 isolate, NIVEDI, Bengaluru, India

ON952534.1:1-420 OvHV2 isolate, Beni Suef, Egypt

MN393474.1:1-393 OvHV2 isolate, Turkey

100

50

40

31

26

23

18

Fig 2: Phylogenetic tree is based on the tegument gene of OvHV-2 outer PCR 422bp nucleotide sequence. Evolutionary analysis was conducted in MEGA 11 Neighbour-joining method with bootstrap consensus tree inferred from 1000 repetitions. The sample VBRI AP in our study is marked with an asterisk

**3.2 Discussion:**

The clinical signs observed in the three affected buffaloes affecting the ocular, respiratory, and digestive systems indicated the possible presence of more than one disease, thereby necessitating conducting multiple tests on same sample. The major viral infections considered were Bovine Viral Diarrhea Virus (BVDV), Malignant Catarrhal Fever (MCF), and Infectious Bovine Rhinotracheitis (IBR), all of which are commonly reported in bovines of Andhra Pradesh. .As noted by Doblies et al. (1998), the most important differential diagnoses in cattle include MCF, mucosal disease(BVD), IBR, foot-and-mouth disease (FMD), and rinderpest. However, with Rinderpest globally eradicated and Andhra Pradesh designated as an FMD-free zone, the diagnostic focus was narrowed to IBR, MCF, and BVDV. Previous reports from the region include cases of sheep-associated MCF, which rarely presents with gastrointestinal symptoms (Neeraja et al., 2023), and a significant prevalence of BVDV, for which diarrhea is a prominent clinical sign (Devi et al., 2023).

Through the application of PCR and sequencing methodologies, the current study has verified that the three buffaloes were concurrently infected with Ovine herpes virus 2 (OvHV-2), the etiological agent of sheep-associated malignant catarrhal fever (MCF), and Border Disease Virus (BDV). BDV, traditionally linked with sheep, is recognized for causing Border Disease, which manifests in clinical symptoms such as abortion, stillbirths, tremors in weak lambs (commonly referred to as "hairy shaker disease"), poor growth, and persistent infections. Animals persistently infected serve as lifelong carriers, disseminating the virus and thereby facilitating its transmission to both sheep and cattle (Romero, et al., 2017). BDV is increasingly being identified in cattle, where it presents with symptoms analogous to those of Bovine Viral Diarrhea Virus (BVDV), including diarrhea, wasting, and abortions. Furthermore, infected cattle have the potential to transmit BDV back to sheep, thereby complicating disease management in mixed-species farming systems (Braun, et al., 2019).

In the current study, PCR amplification and sequence analysis targeting the highly conserved 5’ untranslated region (5’UTR) of the pestivirus genome were employed., This method is widely accepted for differentiating pestiviruses and for classifying them into subgenotypes (Peletto, et al., 2016). Phylogenetic analysis indicated that the pestivirus genome sequence belonged to the BDV-3 subgenotype which was reported in sheep in India byMishra, et al., (2016) who hypothesized that the origin of BDV-3 may be linked to international trade. Further, the sequence was found to cluster distantly from other pestivirus species such as BVDV-1, BVDV-2and the CSFV isolate from Andhra Pradesh. Despite the animals testing positive by pestivirus PCR, the commercial BVDV/BDV antigen ELISA failed to detect the infection.This inconsistency may be attributed to the low sensitivity of the kit (Orr, et al., 1993) or the absence of a specific kit for BDV detection. Therefore BVDV antigen ELISA cannot be used for detection of BDV. Alternative diagnostic methods such as virus isolation or real-time PCR, as recommended by Mishra, et al., (2016) are suggested for accurate detection in field samples.

In the case of MCF caused by OvHV-2, the World Organisation for Animal Health Terrestrial Manual 2018 recommends detection of viral DNA as the confirmatory test. Heminested PCR, which offers greater specificity than conventional PCR, was employed in this study. Sequence analysis revealedclose genetic relatedness to strains previously reported in Andhra Pradesh, southern India from both sheep and cattle.

In both diseases sheep serve as the primary reservoirs. Although mixed farming was not practiced in the current case, mixed grazing of sheep and buffaloes was common in the village. A separate investigation of sheep populations in mixed farming areas in Andhra Pradesh revealed that all 40 tested animals were positive for OvHV-2. These results underscore the critical role of sheep in maintaining and transmitting these viruses and emphasize the need for comprehensive epidemiological surveillance of BDV in regions practicing mixed livestock farming or grazing.

Viral co-infections can lead to three possible interactions: interference, synergy, or no interaction at all (Yanting, et al., 2022). In this instance, viral synergy seems to have intensified the severity of the disease, ultimately resulting in the death of all three animals. Generally MCF is considered as fatal disease but there are several reports where animals survived clinical MCF on multiple occasions (Doblies, et al., 1998) which further confirms the contribution of BDV as a co-infecting virus and its role in aggravating clinical signs should not be underestimated.

Given that sheep act as reservoirs for both viral agents, Implementation of stringent biosecurity protocols and segregation of small ruminants from cattle and buffaloes during grazing or housing is strongly recommended to prevent such cross-species viral transmissions. Field veterinarians should remain vigilant for pestivirus-related symptoms in both species and include BDV in differential diagnoses. Since BDV can also be transmitted from infected cattle to naïve sheep, unchecked circulation of the virus could result in significant economic losses to farmers (Braun, et al., 2019).

**4. Conclusion:** The study determines that the three buffaloes were co-infected with sheep-associated Malignant Catarrhal Fever (MCF) and Border Disease Virus (BDV)as confirmed by clinical symptoms, molecular diagnostics and genetic characterization and death was caused by the synergistic action of both viruses. The sequence analysis revealed that MCF isolate was closely related to other isolates from India and the pestivirus isolate clustered with BDV-3 subgenoytype reported in sheep in India. These findings underscore the critical role of sheep as reservoirs for both infections and highlight the importance of implementing stringent precautions when practicing mixed farming or grazing systems to prevent cross-species transmission.

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