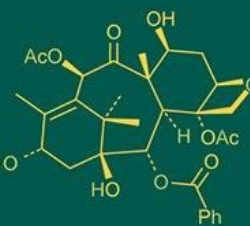
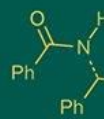
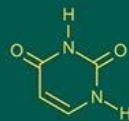
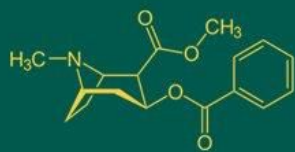


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Glycoproteins C Based Genetic Characterisation of Indian Bovine Herpesvirus-1 with Other Ruminant Herpesviruses

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Abstract

Aim: To characterise immunodominant glycoprotein located on the Unique long (UL44) region of BoHV-1 viz., glycoprotein C (gC) of Indian origin at genetic and phylogenetic level.

Materials and Methods: In this study, archival isolates stored at the ICAR-NIVEDI BSL-2 facility were selected and revived on MDBK cell lines. Partial open reading frames of the gC genes were amplified using conventional PCR, and the resulting sequences were deposited in NCBI. Genome alignment, annotation was performed by GeneTool and phylogenetic analysis by Mega 11 software.

Results: Broadly BoHV-1, BuHV-1, BoHV-5, CeHV-1 formed one cluster and CaHV-1 and RanHV-1 formed another. The phylogenetic analysis of the partial gC gene revealed that all BoHV-1 (including BoHV-1.1 and BoHV-1.2) isolates, including those under study, are highly conserved and grouped within a single clade. In contrast, the bovine herpesvirus 5 (BoHV-5), bubaline herpesvirus-1 (BuHV -1), caprine herpesvirus 1 (CaHV-1), cervine herpesvirus 1 (CvHV-1), and rangiferine herpesvirus 1 (RanHV-1) each formed separate clusters specific to their respective viruses, showing homology ranging from 67.8% to 92.2% with BoHV-1 isolates.

Conclusion: This study will lay the groundwork for future research on BoHV1, particularly in the development of new diagnostic tools.

Keywords: *Bovine herpesvirus* (BoAHV), Glycoprotein C(gC), ruminant herpesviruses, phylogenetic tree

Introduction

Respiratory diseases in bovines, such as Infectious Bovine Rhinotracheitis (IBR) and Bovine Respiratory Disease Complex (BRC), are associated with Bovine Herpesvirus 1 (BoHV-1), a significant pathogen found worldwide (Jefferson *et al.*, 2023) [8]. The virus was first identified in Germany as the causative agent of reproductive disorders like Infectious Vulvovaginitis (IPV). Other conditions it can cause includes Infectious Balanoposthitis (IBP), vision impairment, systemic newborn disease and latent infections in ruminants (Hostnik *et al.*, 2021) [6]. In India, after first isolation of BoHV-1 organism from keratoconjunctivitis cases, both subtypes BoHV-1.1 and BoHV-1.2, have been recovered from IBR, IPV, IBP cases, whose homology was identical to the cooper strain. Serological evidence of IBR has been reported in various species of animals in different parts of the world for example cattle, buffalo, sheep, goats, dromedaries, deer, elephants, hippopotami. Five related herpesviruses, which cause serological cross-reactions with BoHV-1, may interfere with IBR eradication and control programs. These viruses include bovine herpesvirus 5 (BoHV-5), bubaline herpesvirus-1 (BuHV 1), caprine herpesvirus 1 (CaHV-1), cervine herpesvirus 1 (CvHV-1) and rangiferine herpesvirus 1 (RanHV-1) (Ros *et al.*, 1999; Yadav *et al.*, 2024) [21, 26].

Related ruminant herpesviruses are known to cause species-specific diseases. Outbreaks of fatal meningoencephalitis caused by BoHV-5 in calves have been reported in several countries, including Australia, Hungary, the United States, Argentina and Italy. In India, the first case of BoHV-5 was detected in a cattle abortion case in Rajasthan (Kumar *et al.*, 2020) [10]. Initially, BuHV-1 was associated with subclinical disease in water buffalo and the virus has been isolated from the prepuce of buffaloes in Australia, vulvovaginitis cases in India (Yadav *et al.*, 2024) [26], after pharmacological reactivation in Italy, and from abortion cases in the

Mediterranean region (Amoroso *et al.*, 2013)^[1]. CapHV-1, which is globally distributed, causes generalised disease in young kids, primarily affecting the digestive tract, while in adult goats, the infection often remains unnoticed or may cause abortion, vulvovaginitis, or balanoposthitis. It is suspected that sheep and goats might be involved in the transmission of BoHV-1, as the presence of the virus has been detected in Iran, Egypt, and Pakistan. CerHV-1 was first isolated in 1982 from an outbreak of ocular disease on a red deer farm in Scotland and is now widespread in both free-living and farmed red deer. RanHV-1 was isolated from reindeer in Finland. Species differentiation during seroepidemiology is important in proper diagnosis and control strategies.

Glycoprotein C (gC) one among many types I transmembrane glycoprotein, is a non-essential protein, N-terminal forms a spike that interacting with heparan-sulphate on the cell surface contributing to the host cell type tropism (Rashmi *et al.*, 2024)^[19]. It consists of 521 (BoHV-1) amino acid residues that belong to the immunoglobulin superfamily. (Fitzpatrick *et al.*, 1989)^[4]. Both N-terminal and C-terminal regions are highly variable to distinguish the subtypes and amplification of residues from 321 to 450 by PCR region, has often been envisaged as a target for differentiating the subtypes by few authors (Rijsewijk *et al.*, 1999; Esteve *et al.*, 2008)^[20,3]. Similarly in the present study the total of 192 amino acid residues of gC, towards carboxy terminal region were subjected for genome alignment and phylogenetic analysis in relation to other ruminant herpes viruses

Materials and Methods

Collection and Processing of Samples

Five isolates of Bovine alpha-herpesvirus type 1 (BoAHV-1), archived at the ICAR-NIVEDI laboratory from 2010 to 2023 (Table 1), were selected for revival in this study. The virus was propagated using MDBK cell lines on EMEM medium supplemented with 10% Foetal Bovine serum and antibiotic. The cells were regularly observed under an inverted microscope at 6, 24, and 48 hours to monitor the development of cytopathic effects (CPE). Once 90% cell growth was achieved or rounding of cells - characteristic CPE was observed (Fig 1), the virus-inoculated cells were subjected to three cycles of freezing and thawing. Approximately 200 microliters of the cultured virus were then aliquoted and stored at -20 °C until further use

Polymerase chain reaction: Viral DNA was extracted from the cell culture supernatant using the DNeasy Blood & Tissue Kit (QIAGEN, Germany) according to the manufacturer's protocol. The eluted DNA was then stored at -20 °C. For molecular characterization of the isolates, PCR amplification was performed on the partial gC genes. The primers used for this amplification, gC F (560 – GAA GGA GCG CAA GTG GAT GCT CT- 583) and gC R (1138-CGG AGT CGT CGA CCG TAA AGA CGT-1115), target the carboxy-terminal region of the gC gene and were previously designed from the same lab (Ranganath *et al.*, 2013)^[18]. The polymerase chain reaction (PCR) was conducted using a Bio-Rad S1000 Touch PCR Thermal Cycler in a 25 µL reaction mixture within a 200 µL thin-wall PCR tube. Each reaction contained 12.5 µL of Green Taq PCR Master Mix (Thermo Scientific), 0.5 µL of forward primer (10 pmol/µL), 0.5 µL of reverse primer (10

pmol/µL), 5 µL of template DNA, and nuclease-free water to reach a final volume of 25 µL. After centrifugation, the mixture was subjected to a thermal cycling program: initial denaturation at 94 °C for 15 minutes, followed by 34 cycles of denaturation at 94 °C for 50sec, annealing at 65 °C for 50 sec, and extension at 72 °C for 50 sec, with a final extension at 72 °C for 10 minutes. The expected PCR product bands were visualised by running 15 µL of the PCR product on a 1.7% agarose gel and analysing it with a Bio-Rad Gel Doc 2000. All PCR bands of expected size were gel-purified using the NucleoSpin Gel & PCR Clean-up Mini kit (MACHEREY-NAGEL) and subsequently submitted for Sanger sequencing at Indus BioSolutions Ltd, Bangalore, India.

Using forward and reverse primers mentioned above both sense and antisense strands of amplicons were sequenced. The resulting nucleotide sequences were analysed using BLAST, aligned, and annotated with GeneTool, then submitted to GenBank. Accession numbers received for all five sequences, along with details such as origin, year, sex, and sample type from which the virus was isolated, are provided in Table 1. In addition to the sequences from the five isolates in this study (Table 1), sequences of representative bovine herpesvirus isolates from different genotypes (BoAHV-5), and related herpesviruses such as Bubaline herpesvirus, caprine herpesvirus 1 (CaHV-1), cervine herpesvirus 1 (CvHV-1) and rangiferine herpesvirus 1 (RanHV-1) were retrieved from GenBank (Table 2). Multiple sequence alignments were performed using CLUSTALW, followed by construction of the phylogenetic trees based on the maximum likelihood approach according to the Tamura- 3-parameter model using mega11 software (Tamura *et al.*, 2021)^[9].

Results

Virus Isolation and Cell Culture: Five BoAHV-1 isolates collected and stored between 2010 to 2023 were utilised in this study. Monolayers of MDBK cells were inoculated with glycerol stock virus samples for revival following standard protocols (OIE Manual, 2023)^[12]. Microscopic examination of cell monolayers revealed the presence of grape-like clustering within 30 to 48 hours post inoculation (fig 1).

PCR Amplification: The expected product sizes 572 bp for the glycoprotein C genes was amplified and visualised with ethidium bromide staining after agarose gel electrophoresis. The desired bands eluted and sequenced were submitted to NCBI and

Genome alignment and Phylogenetic analysis: Broadly BoHV-1, BuHV-1, BoHV-5, CeHV-1 formed one cluster and CaHV-1 and RanHV-1 formed another. All five partial gC sequences from the present study exhibited 99.7% to 100% similarity among them and close relation to BoAHV 1.1. Genome with a homology of 98.3 to 100% to vaccine strains from USA-Bovishield Gold MLV, Titanium IBR MLV, Switzerland Cooper strain, ATCC Los Angeles strain, previously reported BoAHV 1.1 strains of India and Egypt. Genome identity of 91.6 to 92.0% observed for Brazil and Indian BoHV-5 strains, 92.2% for Bubaline herpes virus, 89.6 to 89.85 for cervid herpes virus of China, Italy, Switzerland., 67.8 to 68% for rangiferin herpes Finland and 66.9 to 67.1% for Caprine herpes virus of Switzerland, Italy, China.

Table 1: Details of BoAHV-1 isolates, place, year, hosts and accession numbers obtained for partial Glycoprotein C(gC) gene.

Sl.no	Isolate ID	Place	Year	Source	Host	Accession number for gC gene
1.	688	Odisha	2010	Vaginal swab	Cow	PP236074
2.	711	Karnataka	2010	Nasal swab	Cow	PP236076
3.	742	Kolkata	2010	Semen	Bull	PP236077
4.	777	Odisha	2011	Nasal swab	Calf	PP236075
5.	11705	Karnataka	2023	Nasal swab	Cow	PP236078

Table 2: Details of Bovine herpesvirus and related virus isolates used in the present study for partial nucleotide and phylogenetic analysis Glycoprotein C(gC) genes.

Sl. No	Accession number	Isolate details	Year of collection	Country
1.	AJ004801	BoAHV 1 complete genome Cooper strain	1956	Switzerland
2.	MH724208	BoAHV 1 BoviShield Gold FP5 MLV vaccine	2013	USA
3.	OR211605	BoAHV 1 isolate 16453/07 TN. complete genome	2007	Italy
4.	MF421714	BoAHV 1 culture ATCC: VR-188 strain Los Angeles, complete genome	1956	USA
5.	KY215944	BoAHV 1 isolate 216 II, complete genome	1976	India
6.	KM258881	BoAHV 1.2 strain B589 complete genome	2001	Australia
7.	KU198480	Bovine alphaherpesvirus 1 strain Cooper, complete genome	1966	USA
8.	JN171865	Bovine herpesvirus 1 isolate PD_ADMAS741/10 glycoprotein C (gC) gene, partial cds	2010	India
9.	JN22593	Bovine herpesvirus 1 isolate PD_ADMAS707/10 glycoprotein C (gC) gene, partial cds	2010	India
10.	JN22594	Bovine herpesvirus 1 isolate PD_ADMAS723/10 glycoprotein C (gC) gene, partial cds	2010	India
11.	OP777904	Bovine alphaherpesvirus 1 isolate Egypt/Sohag/2022 glycoprotein C gene, partial cds	2022	Egypt
12.	MH724203	Bovine alphaherpesvirus 1 isolate Titanium IBR MLV vaccine, complete genome	2013	USA
15.	OP035381	BoAHV1.2, complete genome	2020	China
16.	KM258880	BoAHV 1.2 strain K22.complete genome.	2016	USA
17.	PP897810	Bovine alphaherpesvirus 5 BoHV-5/Cattle/India/2018/Bhilwara, complete genome	2018	India
18.	KY549446	BoAHV 5 strain ISO 97/45 complete genome	1997	Brazil
19.	KY559403	BoAHV 5 strain ISO 97/45. complete genome	1996	Brazil
20.	OQ442798	BuAHV -1 genomic sequence	2012	Italy
21.	NC043054	BuAHV 1 strain b6. complete genome	1972	Australia
22.	NC075564	Caprine alphaherpesvirus 1 strain JSHA1405, complete genome	2023	USA
23.	AF139909	Ragiferin Herpes virus	1999	Sweeden
24.	Z49225	Caprine herpesvirus type 1 gene for glycoprotein gC	1995	Switzerland
25.	KY711176	Caprine alphaherpesvirus 1 strain JSHA1401 glycoprotein gC (gC) gene, complete cds	2014	China
26.	AY821804	Caprine herpesvirus 1 strain Ba-1 from Italy glycoprotein C (gC) gene, complete cds	1996	Italy

**Fig 1:** Neighbour-Joining Tree Generated by 559 bp read-length of Glycoprotein C (UL 44) Region. The figure illustrates the genetic relationship of isolated BoHV 1 virus with selected NCBI-published bovine herpesviruses and related herpesviruses, based on carboxy terminal partial UL 44 (gC) sequences. The numbers at each branch indicate the percentage frequency of this evaluation after 1000 bootstrap evaluations. Labelled isolates represent BoHV 1 Isolates of present study.

Discussion

Bovine herpesvirus-1 is a major pathogen affecting the Bovidae family, which includes cattle, buffalo, yak, and Mithun in India. According to the 20th Livestock Census (dahd), India's total bovine population (cattle and buffalo) stands at 302.34 million. A nationwide seroprevalence study conducted by Patil *et al.* (2023)^[37] using Avidin Biotin (AB) ELISA found a cumulative seropositivity rate of 31.37%, indicating that around 94.84 million animals are affected by the disease. This has a significant economic impact, primarily due to reduced milk production, repeat breeding, abortions, increased feed costs and treatment expenses.

A total of five bovine herpesvirus isolates—three from nasal swabs, one from extended frozen semen, and one from a vaginal swab sample obtained from Cattle—were used in this study. The observation of cytopathic effect was in correlation with previous BoHV-1 isolation reports of Ravishankar *et al.* (2012), Chandrunaik *et al.*, (2014). To determine the subtype of the BHV-1 isolate, a 578 bp segment of the gC gene was amplified via PCR, gel purified, sequenced and submitted to NCBI.

All BoHV 1 isolates, including those under study, clustered within a single clade. Phylogenetic analysis of the complete ORF and partial ORF of the gC gene has been employed by various authors to confirm the prevailing virus subtype of India (Majumder *et al.*, 2013)^[11]. Although the presence of BoHV1.2, BoHV 5, and BuHV 1 has been documented in India, partial carboxy-terminal gC gene analysis of present study indicated that the most commonly occurring subtype is BoHV 1.1, as supported by the findings of Sreenivasa *et al.* (1996)^[22]. The detailed phylogenetic analysis showed that the isolates which were clustered under BoHV-1.1 were genetically 100% close to USA Cooper strain, Titanium IBR MLV vaccine, BoviShield Gold FP5 MLV vaccine and Switzerland strain. Similar findings have been described by various authors using various glycoprotein genes Sreenivasa *et al.* (1996)^[22], Patil *et al.* (2006)^[13] Patil *et al.* (2012)^[14], Majumder *et al.*, (2013)^[11], Chandrunaik *et al.*, (2014)^[2] and Patil *et al.* (2016)^[15]. All other related herpes viruses share at least 67% homology and 33% divergence with BoHV-1, posing challenges for control programmes.

To maintain herd health and minimise financial losses, screening, surveillance, and monitoring are crucial. Among the many three related ruminant herpes viruses, BoHV-1, BoHV-5 and BuHV 1) has been reported from India (Patil *et al.*, 2017; Kumar *et al.*, 2020; Yadav *et al.*, 2024)^[16, 10, 26]. Presently Real time PCR based on gB gC gD gI and gE are used for disease diagnosis from clinical cases (OIE, 2023)^[12]. Indigenous Whole virus antigen based Avidin Biotin ELISA kit has been utilised in seromonitoring process. Identifying highly conserved regions and creating diagnostics to differentiate between related ruminant herpesviruses is crucial for seromonitoring. This study establishes a basis for developing new diagnostic strategies.

The authors declare that they have no conflict of interest.

Conclusion

In conclusion, this study highlights the genetic characterization and phylogenetic relationships of BoHV-1 isolates collected over 13 years. Our analysis revealed that all five isolates clustered within the BoHV-1.1 subtype, showing a high genetic similarity (99.7% to 100%) to strains from the USA and Switzerland. The findings confirm the predominance of the BoHV-1.1 subtype in India, consistent

with previous studies. The genetic homology observed with various vaccine strains underscores the relevance of ongoing surveillance and targeted control measures to manage the economic impact of bovine herpesvirus infections. Effective diagnostic tools and seromonitoring strategies are essential for early detection and prevention of this significant pathogen in ruminant populations.

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