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Molecular Characterisation of US6 (gD) and US8 (gE) Glycoprotein Genes Located on a Unique Short Region of *Bovine Alphaherpesvirus*-1 (BoAHV-1) Genome

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ABSTRACT

Background: *Bovine alphaherpesvirus* type 1 (BoAHV-1) is a significant pathogen causing respiratory diseases and reproductive issues in cattle and buffaloes, resulting in substantial economic losses globally. This study provides a comprehensive analysis of the genetic characteristics of BoAHV-1 isolates from India, focusing on glycoprotein genes US6 (gD) and US8(gE).

Methods: Ten archival isolates that were stored at the ICAR-NIVEDI BSL-2 facility were chosen, revived on MDBK cell lines and subjected for molecular characterization through PCR amplification of partial US6 (gD) and US8 (gE) gene. Genome alignment and phylogenetic analysis were performed by GeneTool and Mega 11.

Result: Phylogenetic analysis of both genes revealed two cluster formation, BoAHV-1.1, BoAHV-1.2 in one cluster and BoAHV-5, *Bubaline alphaherpesvirus* (BuAHV-1) in another cluster. All ten isolates displayed close relation to BoAH 1.1. Seven isolates from each phylogenetic analysis showed 100% genome identity to vaccine strains from USA- Bovishield Gold MLV, Pyramid IBR MLV, Switzerland Cooper strain, ATCC Los Angeles strain, previously reported BoAHV 1.1 strains of India and Egypt. Genome identity of BoAHV-1.1 against BoAHV-1.2 was 97.70 to 98.70% for gD gene and 99.0 to 99.70% for gE gene respectively. The analysis highlights the higher genome identity among BoAHV-1 subtypes in the gE gene, suggesting its potential for developing more sensitive immunoassays for seroepidemiology. The developed assay could also be exploited for detecting BoAHV-5 infections. The findings contribute to the knowledge base essential for development of companion ELISA for implementing Differentiating Infected from Vaccinated Animals (DIVA) vaccination strategies.

Key words: *Bovine alphaherpesvirus* (BoAHV), Phylogenetic tree, US6 (gD), US8 (gE) glycoproteins.

INTRODUCTION

Bovine alphaherpesvirus type 1 (BoAHV-1) belongs to the family *Herpesviridae,* subfamily *Alphaherpesvirinae* and class *Varicellovirus.* Its core comprises a linear 135 kbp, double-stranded DNA genome safeguarded by 162 capsomers in an icosahedral symmetry (Jefferson *et al.,* 2023). Viral glycoproteins encoded by BoAHV genes are organised into a single unique long unit (UL) and a single unique short unit (US), flanked by two inverted repeat sequences known as internal repeat and terminal repeat. These viral glycoproteins, located in the envelope on the virion surface, play pivotal roles in pathogenesis and immunity. Serologically related viruses causing either Infectious Bovine Rhinotracheitis (IBR) or Infectious Pustular Vulvovaginitis (IPV) are collectively referred to as *Bovine alphaherpesvirus-1* (Gibbs and Rweyemamu, 1977), which are further classified into two genotypes: BoAHV-1.1 and BoAHV-1.2. BoAHV-1.2 is subdivided into BoAHV-1.2a and BoAHV-1.2b subtypes (Metzler *et al*., 1985; Ranganatha *et al*., 2013; Zhou *et al.,* 2020). Another closely related virus, BoAHV-5, causes sporadic infections and higher mortality in calves due to encephalitis rarely reproductive infections. In India, BoAHV-5 has been isolated from reproductive tracts but not from cases of encephalitis (Kumar *et al*., 2020). Among the various glycoproteins found on the envelope, gB(UL27), gD(US6) and gC(UL44) are essential, while gE(US8) non-essential for virus replication (Rashmi *et al*., 2024). However, all of these glycoproteins are highly immunogenic in nature. PCR assay or ELISA developed targeting these glycoproteins are used in diagnostics (OIE, 2023).

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Although rarely a very deadly illness, IBR can cause significant economic losses through abortion, loss of body condition, decreased milk output, death of newborn calves, temporary failure of conception, poor feed conversion, subsequent bacterial pneumonia and expensive treatment. Losses in large milking herds have been estimated to range from $25 to $55 per cow (Townley, 1971). Mortality rates in dairy herds are around 3%, whereas in feedlots, they can range from 20-30% and sometimes reach as high as 100%, as reported in Hungary (Barenfus *et al*., 1963). The reproductive form of the illness has lowered the conception rate from 80% to 45-50% in artificially inseminated cows (Laveso *et al*., 1984). Townley (1971) documented an acute outbreak of the respiratory type of IBR in a dairy herd, which had a 30% morbidity rate and resulted in a $500 loss. During an epidemic of the disease's nerve form, Australia experienced a 30% death rate and a 90% morbidity rate (Gardiner *et al*., 1964). Additionally, an annual economic loss of €62 million is attributed to IBR, affecting 80% of the dairy industry in Ireland (Hanrahan *et al*., 2020). In India such economic loss estimates has not yet made but the five year cumulative seroprevalence of 31.37% from twenty five states and three union territories depicts the substantial economic loss (Patil *et al*., 2022).

Glycoprotein D (US6), in conjunction with gB and the gH-gL complex, facilitates the viral fusion process. It is noteworthy that gD is the primary inducer of high neutralizing antibody titres against BoAHV, making it a pivotal vaccine candidate (Dubuisson *et al*., 1992). With a molecular weight of 71 kDa, glycoprotein D exhibits a 79.9% amino acid identity between BoAHV-1 and BoAHV-5 (Delhon *et al*., 2003). ELISA based on gD are used to identify the infection. On the other hand, the association of gE with gI and US 9 sequence protein facilitates the axonal sorting and reverse axonal transport of viruses in neurons (Ning *et al*., 2022). Deletion of the US8 do affect the cell-to-cell movement of virus but not the viral copy number. Further gE deleted vaccines and ELISA based on gE are commonly used to differentiate infected from vaccinated animals in disease control programmes (Petrini *et al*., 2020).

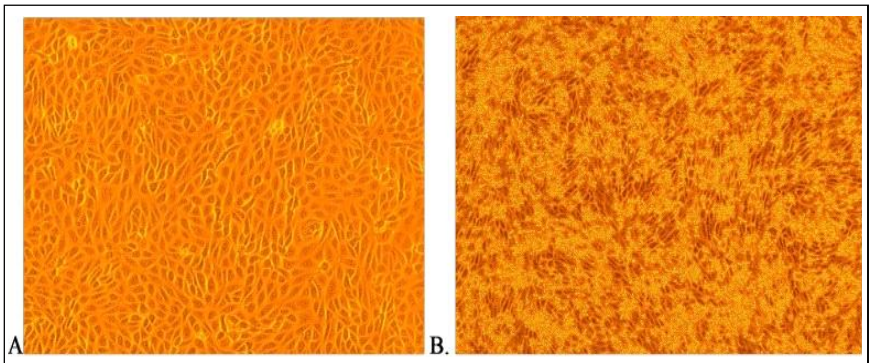
MATERIALS AND METHODS

Collection and processing of samples

Ten isolates of *Bovine alpha-herpesvirus type 1* (BoAHV-1), archived at ICAR-NIVEDI laboratory over a 12-year period (Table 1), were chosen for revival in this study. The MDBK cell lines to propagate the virus were obtained from NCCS Pune. Cells were regularly observed under an inverted microscope at 6, 24 and 48 hours to monitor the cytopathic

Table 1: Details of BoAHV-1 isolates, place, year, hosts and accession numbers.

Isolate ID	Place	Year	Source	Host	Accession number for gE gene	Accession number for gD gene
685	Odisha	2010	Nasal swab	Cow	PP211003	PP211013
688	Odisha	2010	Vaginal swab	Cow	PP211004	PP236068
711	Karnataka	2010	Nasal swab	Cow	PP211005	PP211014
742	Kolkata	2010	Semen	Bull	PP211006	PP211015
743	Pune	2010	Semen	Bull	PP211007	PP211016
744	Pune	2010	Semen	Bull	PP211008	PP236069
746	Pune	2010	Semen	Bull	PP211009	PP236070
777	Odisha	2011	Nasal swab	Calf	PP211010	PP236071
926	Odisha	2011	Blood	Bull	PP211011	PP236072
11705	Karnataka	2023	Nasal swab	Cow	PP211012	PP236073



A. healthy cell lines at 0 hrs. B. grape like clustering of cells at 36 hrs.

Fig 1: Cytopathic effect of BoAHV-I observed in MDBK cell lines at 0 and 36 hours of post infection.

effects (CPE) development. Once 90% growth was achieved or characteristic CPE was observed (Fig 1), the cells inoculated with virus samples were subjected for three cycles of freezing and thawing. Around 200 microliter of cultured virus were subsequently aliquoted and stored at -20°C until use.

Polymerase chain reaction

Viral DNA extraction from cell culture supernatant was performed using the DNeasy Blood and Tissue Kit (QIAGEN, Germany) following the manufacturer's instructions. The eluted DNA was stored at -20°C. Molecular characterization of the isolates involved PCR amplification of the partial gD and gE genes. Details of the primers used and their respective product sizes are provided in Table 2. Polymerase chain reaction (PCR) was carried out using a Bio-Rad S1000 Touch PCR Thermal Cycler in a 25 µl total volume reaction mixture in a 200 µL capacity thin-wall PCR tube. Each reaction consisted of 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 25.0 µL. The resulting mixture, after centrifugation, underwent a precise thermal profile as follows: initial denaturation at 95°C for 10 minutes, followed by 29 cycles at 95°C for 1-minute denaturation, specific annealing temperatures of 60°C for gE and 61°C for gD and extension at 72°C for 1.5 minutes, concluding with one cycle at 72°C for 10 minutes. The expected band sizes were visualised using a Bio-Rad Gel Doc 2000 after running 15 µL of PCR product on a 1.7% agarose gel. All PCR amplicons were gel-purified (QIAquick® Gel Extraction Kit, Qiagen, USA) and submitted for sequencing.

Phylogenetic analysis

Following gel purification, gE and gD gene fragments were subjected for Sanger sequencing at Indus BioSolutions Ltd, Bangalore, India, using the forward and reverse primers listed in Table 2. The nucleotide sequences were BLAST analysed, aligned using GeneTool and submitted to GenBank. Generated Accession numbers of all twenty sequence were listed in Table 1. In addition to the sequences of the twenty isolates from the present study (Table 1), sequences of representative bovine herpesvirus isolate of different genotypes, BoAHV-5 and related herpesviruses such as Bubaline herpesvirus, were retrieved from GenBank (Table 3). Multiple sequence alignments were performed using CLUSTALW followed by Neighbor-Joining tree to assess phylogenetic relationships with various lineages using Mega 11.

RESULTS AND DISCUSSION

Virus isolation and cell culture

BoAHV-1 isolates collected and stored between 2009 to 2023 were utilised in this study. Monolayers of MDBK cells were inoculated with glycerol stock virus samples for revival following standard protocols (OIE, 2023). Microscopic examination of cell monolayers revealed the presence of grape-like clustering within 30 to 48 hours post infection (Fig 1).

PCR amplification

The expected product sizes of 343 bp (Fig 4) and 652 bp (Fig 5) for the US6 (glycoprotein D) and US8 (glycoprotein E) genes respectively were amplified and visualised with ethidium bromide staining after agarose gel electrophoresis.

Genome alignment and phylogenetic analysis of US6 gene

All partial US 6 (gD) sequences from the present study exhibited 99.7% to 100% similarity among them and close relation to BoAHV 1.1. Seven isolates showed 100% nucleotide identity with vaccine strains from the USA, including Bovishield Gold, Pyramid IBR MLV, Switzerland cooper strain, ATCC Los Angeles strain, previously reported BoAHV 1.1 strains of India and Egypt. The remaining three virus isolates (688, 742 and 744) displayed identity ranging from 99.3% to 99.7% forming a sub branch in the phylogenetic tree, however no difference in open reading frame was observed. Variable levels of identity were observed with BHV-1.2 strains (ranging from 96.8% to 98.7%), BoAHV-5 (89.9% to 90.5%) and BuHV-1 (90.5% to 91.2%). Overall, the least genome identity was observed against the BoAHV5 strains (Fig 2).

Genome alignment and phylogenetic analysis of US8 gene

All isolates of present study displayed close relation to BoAHV 1.1 subtype, similar to the US6 gD gene. The nucleotide sequence alignment of the partial US8 (gE) gene of the seven virus isolates and published sequences, including Bovishield Gold, Pyramid IBR MLV, Switzerland cooper strain, ATCC Los Angeles strain, previously reported

Table 2: Details of oligonucleotides used for the amplification of glycoprotein genes.

Target gene	Nucleotide sequence (5′-3′)	Product size (bp)	Reference
US6 (gD)	For- GTCGAGGTCCGCTACGCGACG Rev-CCGCGAGCCGCGCCGAGTTTGCA	343	Smits *et al.*, 2000
US8 (gE)	For-AAACCCCGCATATGGCTTCGGT CGACACGGTCTTCA Rev-GTCGAAGGAT CCAGACTGCAGACGCACCGATAG	652	Oliveira *et al.*, 2013

BoAHV 1.1 strains of India, Italy, China, Egypt, revealed 100% homology. Three virus isolates (685, 746 and 11705) exhibited variable percentages of homology (ranging from 99.3% to 99.5%). In case of 11012 virus, at 578 nucleotide position nucleotide C was present instead of T, leading to change in the amino acid to P (Proline) instead L (Leucine). However, isolate 685 and 746 though exhibited nucleotide change at 591 positions, no amino acid change was found. The levels of genomic similarity ranged from 99.2% to 100% for BoAHV-1 isolates (n=10), 99.00% to 99.7% between BoAHV-1.2 reference isolates, 89.00% to 89.7% for BoAHV-5 and 90.08% to 91.03% between BuAHV 1. Bubaline herpesviruses were found to be closer to BoAHV-1 than to BoAHV-5 (Fig 3).

In this study, *Bovine alphaherpesvirus* samples obtained from the repository of the ICAR- NIVEDI Virology lab, isolated from various sources such as nasal swabs, vaginal swabs, blood *and* semen samples from cattle, were successfully revived on MDBK cell lines following established protocols. Among the ten virus isolates studied, four originated from Orissa, three from Pune, two from Karnataka and one from Kolkata. Confirmation of BoAHV-1 presence was achieved through the display of characteristic cytopathic effects and amplification of the gD and gE genes, with expected product sizes of 343 bp and 652 bp, respectively (Fig 4 and 5).

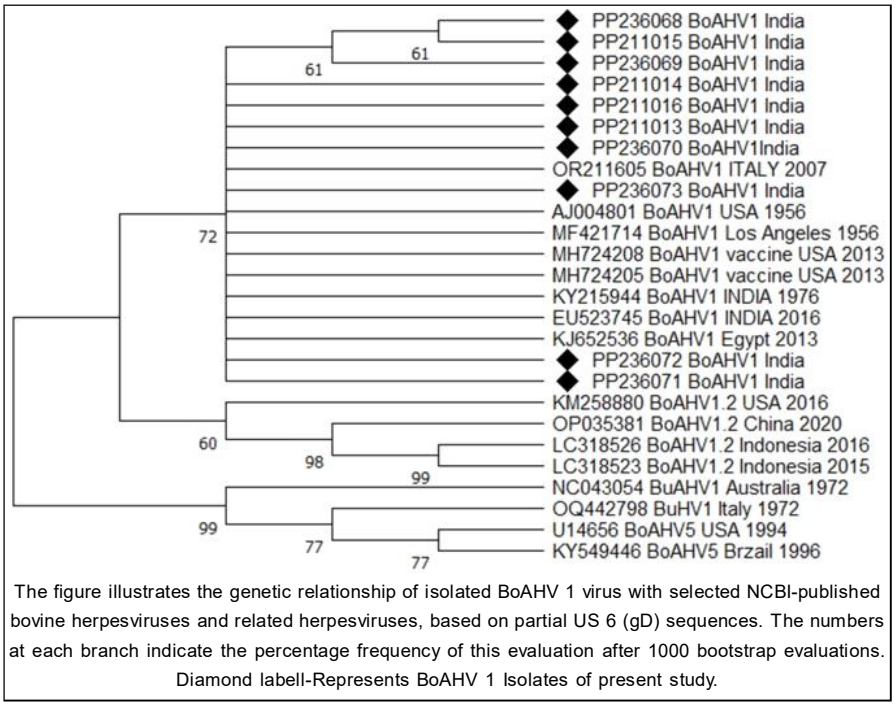
All twenty PCR-amplified partial gene sequences (ten gE and ten gD) underwent direct sequencing and phylogenetic analysis using the Neighbour joining method. Partial nucleotide sequence analysis of the essential US6 (gD) gene and non-essential US8 (gE) gene allowed for the determination of genetic relatedness of the isolates with other herpesvirus strains from worldwide. The analysis revealed two clusters of the virus, with all ten isolates grouped as BoAHV-1.1, along with various reference BoAHV-1 and BoAHV-1.2 strains. Related herpes viruses such as BoAHV 5 and BuAHV formed separate cluster. This finding is consistent with previous studies by other authors, albeit based on different glycoprotein genes gB (Patil *et al*., 2012; Chandranaik *et al.,* 2014; Surendra *et al.,* 2015; Patil *et al.,* 2016; Kumar *et al.,* 2020; Guo *et al.,* 2022), gC (Fitzpatrick *et al*., 1989; Sivarama *et al*., 1999; Esteves *et al.,* 2008; Surendra *et al.,* 2015; Kumar *et al.,* 2020; Guo *et al.,* 2022) and gD (Surendra *et al.,* 2015; Noor *et al.,* 2019; Guo *et al.,* 2022). Clustering of all BoAHV 1.1 Indian isolates with Cooper strain observed in the present study was supported by other previous studies (Patil *et al.,* 2006; Patil *et al.,* 2012; Patil *et al.,* 2016; Chandranaik *et al*., 2014). In contrast

Table 3: Details of bovine herpesvirus and related virus isolates used in the present study for partial nucleotide and phylogenetic analysis US 6 and US8 genes.

Accession number	Isolate details	Year of collection	Country
AJ004801	BoAHV 1 complete genome Cooper strain	1956	Switzerland
MH724208	BoAHV 1 BoviShield Gold FP5 MLV vaccine	2013	USA
OR211605	BoAHV 1 isolate 16453/07 TN. complete genome	2007	Italy
MF421714	BoAHV 1 culture ATCC:VR-188 strain Los Angeles, complete genome	1956	USA
EU850282	BoAHV 1.1 strain Abu-Hammad truncated gE gene. Complete cds.	2013	Egypt
KY215944	BoAHV 1 isolate 216 II, complete genome	1976	India
KM985498	BoAHV 1 glycoprotein E gene, complete cds	2011	India
GU591891	BoAHV 1 strain NM06 glycoprotein E (US8) gene		China
KM258881	BoAHV 1.2 strain B589 complete genome	2001	Australia
OQ442798	BuAHV -1 genomic sequence	2012	Italy
KY559403	BoAHV 5 strain ISO 97/45. complete genome	1996	Brazil
AF208294	BoAHV-5 gE complete genome	2000	USA
KJ652536	BoAHV-1.1/Abu-Hammad/2013/2/Egypt glycoprotein D (gD) gene, partial cds	2013	Egypt
OR211605	BoAHV1 1 isolate 16453/07 TN complete genome	2007	Italy
EU523745	BoAHV-1PD_ADMAS-1 gD gene	2008	India
KY215944	BoAHV 1 isolate 216II. complete genome	1976	India
MH724205	BoAHV 1 isolate Pyramid IBR MLV vaccine	2013	USA
MH724208	BoAHV 1 isolate Bovishield goldFP5 MLV vaccine	2013	USA
LC318523	BoAHV 1 US6 gene strain 1/Bovine/Bukittinggi/ Indonesia/2015/	2015	Indonesia
LC318526	BoAHV 1 US6 gene strain 9/Bovine/Lampung/ Indonesia/2016	2016	Indonesia
OP035381	BoAHV 1.2, complete genome	2020	China
KM258880	BoAHV 1.2 strain K22.complete genome.	2016	USA
U14656	BoHV 5 gD gene complete cds	1994	USA
KY559403	BoAHV 5 strain ISO 97/45. complete genome	1996	Brazil
KY549446	BoAHV 5 strain ISO 97/45 complete genome	1997	Brazil
NC043054	BuAHV 1 strain b6. complete genome	1972	Australia

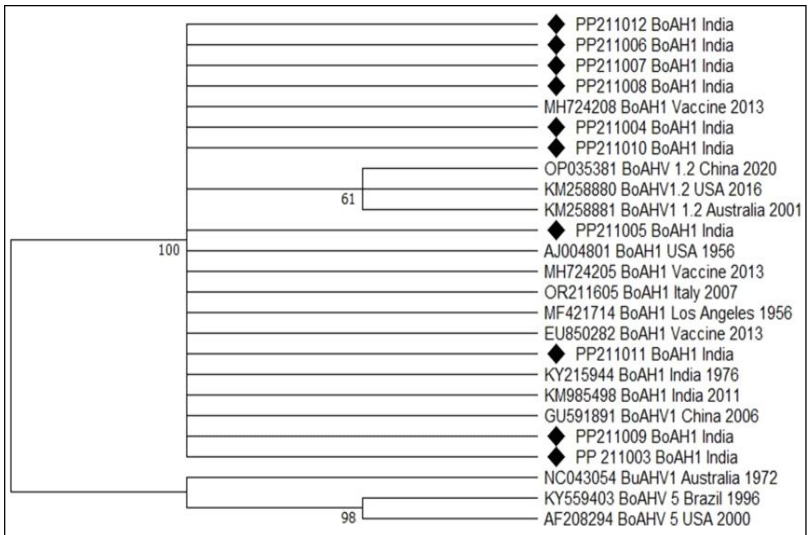
to the tree based on the US8 (gE) gene, the phylogenetic tree based on the US6 (gD) gene showed a clearer branching of BoAHV 1.2 from BoAHV 1.1. This variation is explained by the increased subtype polymorphism, especially in the US6 gene. The observation contradicted a comparative amino acid analysis of the US 6 (gD) and UL36 genes of Indonesian BoAHV-1.2 field isolates, which found that subtype polymorphism was higher in the UL36 area than in the gD region (Noor *et al*., 2019). Further, higher genome identity among subtypes in the US8 gene could indeed contribute to amino acid identity and development of more sensitive immunoassays for sero-epidemiology.

In India, numerous serological and molecular epidemiological studies conducted since 1976 have



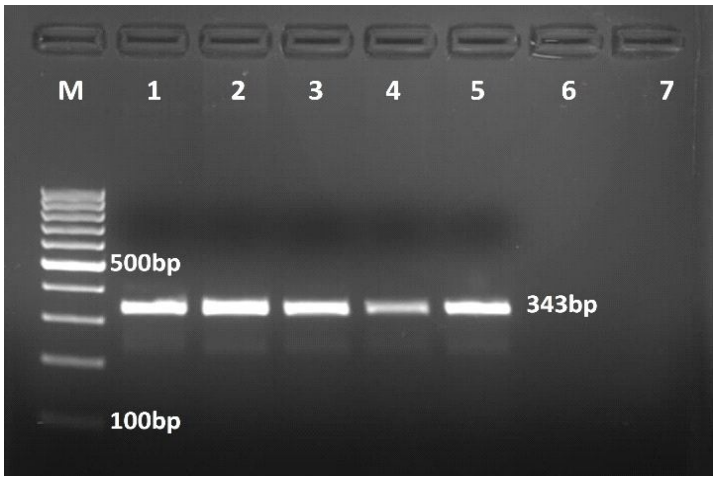
The figure illustrates the genetic relationship of isolated BoAHV 1 virus with selected NCBI-published bovine herpesviruses and related herpesviruses, based on partial US 6 (gD) sequences. The numbers at each branch indicate the percentage frequency of this evaluation after 1000 bootstrap evaluations. Diamond labell-Represents BoAHV 1 Isolates of present study.

Fig 2: Neighbour-Joining tree generated by 300 bp read-length of glycoprotein D (US 6) region.



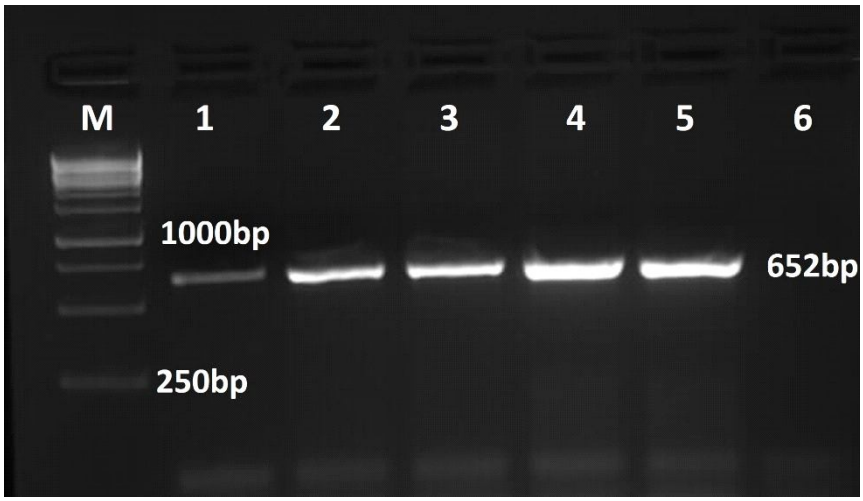
The figure displays the genetic relationship of isolated BoAHV 1 virus with selected NCBI-published bovine herpesviruses and related herpesviruses, based on partial US 8 (gE) gene sequences. The numbers at each branch represent the percentage frequency of this evaluation after 1000 bootstrap evaluations. Diamond label -Represents BoAHV 1 Isolates of present study.

Fig 3: Neighbour-joining tree Generated by 603 bp read-length of glycoprotein E (US 8) region.



Lane M : 100bp DNA marker, Lane 1: Positive control, Lane 2, 3,4,5: BoAHV-1 gD, Lane 6: Negative control, Lane 7: Non template control.

Fig 4: Amplified PCR prodect of Glycoprotein D (343bp) separated in 1.5% agarose gel electrophoresis.



Lane M : 1kb DNA marker, Lane 1 : Positive control, Lane 2, 3,4,5,: BoAHV-1 gE, Lane 6: Negative control.

Fig 5: Amplified PCR prodect of glycoprotein E (652 bp) separated in 1.5% agarose gel electrophoresis.

confirmed the endemic nature of BoAHV infection across almost all states and union territories (Patil *et al*., 2017; Rashmi *et al.,* 2024). While testing and culling of infected animals have been implemented in bull mother farms, widespread virus control through vaccination and differentiation of vaccinated from infected animals remains limited. Understanding the prevalent strains and mutations within these strains is imperative for effective disease control programs. The implementation of the Differentiating Infected from Vaccinated Animals (DIVA) vaccination strategy, necessitates the use of glycoprotein-E based immune assays to differentiate between infection and vaccination status. In this aspect the findings of current studies suggest that, the higher genome identity observed among BoAHV 1 subtypes in the US8 gene could facilitate the development of more sensitive immunoassays for sero-epidemiology, which might cross detect BoAHV 5 and also BuAHV.

CONCLUSION

IBR caused by BoAHV-1 is endemic India and is one of the important diseases that needs immediate attention in bull mother farms/semen collection centres. Isolation of the virus is very difficult as the virus remains latent in infected animals. The isolates used in this study were characterised by nucleotide sequencing and all of them belonged to BoAHV-1.1. Seven isolates from each phylogenetic analysis showed 100% genome identity to vaccine strains from USA-Bovishield Gold MLV, Pyramid IBR MLV, Switzerland Cooper strain, ATCC Los Angeles strain, previously reported

BoAHV 1.1 strains of India and Egypt. The analysis highlights the higher genome identity among BoAHV-1 subtypes in the gE gene, suggesting its potential for developing more sensitive immunoassays for seroepidemiology.

Conflict of interest

The authors declare that they have no conflict of interest.

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