Isolation and characterization of bovine herpes virus 5 (BHV5) from cattle in India

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Abstract

Bovine herpesvirus 1 (BHV1) and 5 (BHV5) are genetically and antigenically related alphaherpesviruses. Infection with one virus induces protective immunity against the other. However, disease associated with BHV1 and BHV5 varies significantly; whereas BHV1 infection is usually associated with rhinotracheitis and abortion, BHV5 causes encephalitis in cattle. BHV5 outbreaks are sporadic and mainly restricted to the South American countries. We report BHV5 infection for the first time from aborted cattle in India. Based on the characteristic cytopathic effects in MDBK cells, amplification of the viral genome in PCR, differential PCR for BHV1/BHV5, nucleotide sequencing and restriction endonuclease patterns, identity of the virus was confirmed as BHV5 subtype A. Serum samples from the aborted cattle strongly neutralized both BHV1 and BHV5 suggesting an active viral infection in the herd. Upon *UL27*, *UL44* and *UL54* gene-based sequence and phylogenetic analysis, the isolated virus clustered with BHV5 strains and showed highest similarity with the Brazilian BHV5 strains.

Author Summary

BHV5 causes fatal meningoencephalitis that may result in a mortality rate of up to 100% in cattle. BHV5 is rarely associated with abortion and its distribution is restricted to South American countries. Only a few cases of this disease have been reported from other countries. For the first time, we provide a strong evidence of BHV5 infection from aborted cattle in India. The finding may necessitate inclusion of BHV5 test protocol in testing of semen for sexually transmitted diseases. Also, the isolated virus would be useful for developing diagnostic, prophylactic and therapeutic agents to combat BHV5 disease in the country.

Introduction

Bovine herpesvirus types 1 (BHV1) and 5 (BHV5) belong to the family *Herpesviridae*, subfamily *Alphaherpesvirinae* and genus varicellovirus [1]. They are genetically and antigenically closely related and share ~85% nucleotide identity. BHV1 is prevalent globally and causes infectious bovine rhinotracheitis (IBR) in cattle which is characterized by conjunctivitis, rhinotracheitis, balanopostitis, infectious pustular vulvovaginitis, enteritis, abortion and sometimes encephalitis. BHV1 infection results in considerable economic losses due to decreased milk production, weight loss and abortions.

BHV5 usually infects young calves and mortality can reach up to 100% [2]. BHV5 mainly causes fatal meningoencephalitis in cattle and establishes latency in trigeminal ganglion. The virus is excreted in nasal, ocular and genital secretions upon reactivation (under stress). The clinical signs in affected cattle include depression, anorexia, weakness followed by neurological signs, such as incoordination, muscular tremor, blindness, circling, recumbency, head pressing, convulsions, paddling and death [3]. Occasionally, BHV5 has been shown to be associated with the reproductive disorders [4].

Herpesvirus associated bovine meningoencephalitis was first time reported in 1962 in Australia. Based on the virion morphology, cytopathic effect in cell culture and antigenic properties, the isolated virus was initially considered to be a neuropathogenic variant of BHV1, called bovine encephalitis herpesvirus (BEHV) [5] or BHV1 subtype 3 [1]. However, later on, based on the restriction site mapping of viral DNA and cross reactivity with monoclonal antibodies, BEHV was found to be a distinct strain with different genomic and antigenic properties. Thus, in 1992, International Committee on Taxonomy of Viruses recognised BEHV as a distinct virus species, namely BHV5 [6].

The prevalence of BHV5 is not precisely known because the available serologic tests do not discriminate antibodies against BHV1- and BHV5. Naturally occurring or vaccine-

induced BHV1 antibodies confer cross protection against BHV5, a possible reason of non-occurrence of BHV5-associated disease in BHV1 endemic areas [7,8].

BHV5 is endemic in South American countries-Argentina [9], Uruguay [10] and Brazil [11]. Only a few cases of the disease have been reported in Australia [12], Hungary [13], Iran [14], Canada [15] and United States [16]. BHV5 has not been reported in India. We isolated and characterized BHV5 for the first time from aborted cattle in India.

Materials and Methods

Ethics Statement

The study involves collection of biological specimens from cattle (field animals). Vaginal swabs and blood samples (3 ml each) were collected from three aborted and three apparently healthy cattle as per the standard practices without using anaesthesia. ICAR-National Research Centre on Equines, Hisar (India) granted the permission to collect the biological specimens. A due consent was also taken from the farmer (animal owner) before collection of the specimens.

Cells and virus

Madin-Darby bovine kidney (MDBK) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with antibiotics and 10% fetal calf serum. Reference BHV1 (VTCCAVA14) was available in our repository at National Centre for Veterinary Type Cultures (NCVTC), Hisar, India which has been described elsewhere [17,18].

Clinical specimens

The samples originated from an organized cattle farm located near Bhilwara, Rajasthan, India (25.3407° N, 74.6313° E). Out of a total of 68 animals, 61 (including 2 bulls) and 7 (including one bull) belonged to Gir and Tharpakar breeds, respectively. First evidence of abortion in the farm was noticed about 2 years prior to the sampling. Over 25 abortions had

already occurred. Three cattle with a recent history of abortion (3 -18 days prior to sampling) as well as 3 apparently healthy animals without any history of abortion were considered for sampling. Abortions occurred between 4-9th months of pregnancy without any specific pattern. No involvement of nervous system was recorded in any of the aborted cows. The farmer practiced natural service in the farm and never performed artificial insemination for breeding purpose. Besides six-monthly vaccinations against foot-and-mouth disease (FMD), the farmer also employed RB51 calfhoo vaccination against *Brucella*. Bulls for breeding were purchased from Surendranagar, Gujarat, a nearby state (India).

Vaginal swabs were collected in Minimum Essential Medium (MEM, transport medium) and transported on ice. The biological specimens were processed as per the standard procedures described elsewhere [19]. An aliquot of 500 µl suspension of vaginal swab was used for bacteriological examinations. The remaining sample was filtered through 0.45 µm syringe filters and used for various virological assays. Serum/blood samples (3 ml from each animal) were also collected from aborted as well as healthy animals. A due consent was taken from the animal owner for collection of the biological specimens.

Identification of the agent(s)

Initially, the DNA was extracted from vaginal swabs by DNeasy Blood & Tissue Kits (Qiagen, Valencia, CA, USA) and subjected to amplification of the BHV1, *Campylobacter* spp, *Brucella* spp, *Leptospira* spp, *Listeria* spp and *Trichomonas vaginalis* genome, the agents which are commonly associated with abortion in cattle. Primers, annealing temperatures and extension times for amplification of these agents are given in **Table 1**. For PCR amplification, each reaction tube of 20 µl contained 10 µl of Q5 High-Fidelity 2× Master Mix (New England BioLabs Inc.), 20 pmol of forward and reverse primer, and 5 µl of DNA (template). Thermocycler conditions included: a denaturation step of 5 min at 98°C followed by 35 cycles of amplification [(30 sec at 98°C, 30 s at 58-65°C (**Table 1**) and 40-90

s (**Table 1**) at 72°C], and a final extension step at 72°C for 10 min. The PCR products were separated in a 1% agarose gel.

Virus isolation

For virus isolation, an aliquot of a biological specimen (500 µl filtrate) was used to infect a confluent monolayer of MDBK cells for 2 h followed by addition of fresh growth medium. The cells were observed daily for appearance of the cytopathic effect (CPE).

Table 1: Primers used to amplify various agents involved in abortion

Agent	Target Gene	Nucleotide sequences of the oligos	Product size (bp)	Thermocycler conditions	Reference
	UL27 (gB)*	Forward: 5'-CACGGACCTGGTGGACAAGAAG-3' Reverse: 5'-CTACCGTCACGTGAGTGGTACG-3'	484	Annealing = 58°C Extension = 40 s	[20]
	UL27 (gB)#	Forward: 5'- CGGCACGCTCGAACGGCAT -3' Reverse: 5'- AGCAGCTCGTTGCTCTCG -3'	534	Annealing = 58°C Extension = 40 s	[21]
		BHV5-Forward: 5'- CGGACGAGACGC CCT TGG -3' BHV1-Forward: 5'- CAACCGAGACGGAAGCTCC -3'	159	Annealing = 58°C	
BHV1/5	UL44 (gC)\$	BHV1/5-Reverse: 5'- AGTGCACGTACAGCGGCTCG -3'	354	Extension = 30 s	[22]
	UL44 (gC)\$	Forward: 5'- ATGGGCGCTGGGGCGAGC -3' Reverse: 5'- CTACAGGCGCGCCCGGCCTTG -3'	1461	Annealing = 65°C Extension = 90 s	NA
	UL54	Forward: 5'- TACTGCGCGCACTCGGGTAC -3' Reverse: 5'- AGACGCTCATGGTCCACGGC -3'	649	Annealing = 58°C Extension = 40 s	NA
Brucella spp	Omp2b	Forward: 5'- GCGCTCAGGCTGCCGACGCAA -3' Reverse: 5'- ACCAGCCATTGCGGTCGGTA-3'	192	Annealing = 58°C Extension = 30 s	[23]
Listeria spp	hly	Forward: 5'- CCTAAGACGCCAATCGAA -3' Reverse: 5'- AAGCGCTTGCAACTGCTC -3'	702	Annealing = 50°C Extension = 60 s	[24]
Leptospira spp	16s	Forward: 5'- GGCGGCGCGTCTTAAACATG -3' Reverse: 5'- TCCCCCATTGAGCAAGATT -3'	329	Annealing = 53°C Extension = 60 s	[25]
Campylobacter spp	Aspartate kinase	Forward: 5'- GGTATGATTCTACAAAGCGAG -3' Reverse: 5'- ATAAAGACTATCGTCGCGTG -3'	503	Annealing = 48°C Extension = 60 s	[26]
Trichomonas vaginalis	B-tubulin	Forward: 5'- CATTGATAACGAAGCTCTTACGAT -3' Reverse: 5'- GCATGTTGTCCGGACATAACCAT -3'	112	Annealing = 48°C Extension = 60 s	[27]

*Primers for initial diagnostic PCR, #Primers for BHV1/BHV5 differential PCR, \$ Primers for BHV5 subtyping, s=seconds, bp=base pairs, NA=Not applicable (self designed primers)

Plaque assay

Plaque assay was performed as per previously described methods along with some modifications [28,29]. The confluent monolayers of MDBK cells, grown in 6 well tissue

culture plates, were infected with 10-fold serial dilutions of virus for 1 h at 37°C, after which the infecting medium was replaced with an agar-overlay containing equal volume of 2X L-15 medium and 2% agar. The plaques were visible 5-6 days following infection (dpi). The agar-overlay was removed, and the plaques were stained by 1% crystal violet. The viral titres were measured in plaque forming unit/ml (pfu/ml).

Virus neutralization assay

Virus neutralization assay was carried out to evaluate the antiviral antibodies in the infected animals. Serum samples were first heated at 56°C for 30 min to inactivate the complements. MDBK cells were grown in 96 well tissue culture plates at ~ 90 confluency. Two-fold serum dilutions (in 50 µl volume) were made in phosphate buffer saline (PBS) and incubated with equal volume of either BHV1 or BHV5 (10³ PFU/ml) for 1 h. Thereafter the virus-antibody mixtures, along with mock-infected control were used to infect MDBK cells. The cells were observed daily for appearance of CPE for determination of the antibody titres.

Biotyping of BHV5

Based on the restriction endonuclease patterns, BHV5 has 3 subtypes, viz., A, B and C [22,30]. We subjected BHV5 to multiplex amplification of *UL27* and *UL54* genes by PCR followed by digestion by *BstEII*. The banding pattern generated was used to determine biotype of the BHV5 as described previously [22,30].

Nucleotide sequencing

In order to further confirm the identity of the virus, *UL27*, *UL44* and *UL54* genes were amplified by PCR, gel purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and subjected to direct sequencing using both forward and reverse PCR primers. Duplicate samples were submitted for sequencing and high-quality sequences were deposited in the GenBank database with an Accession Numbers viz; MN852441 (*UL27*), MN852442 (*UL44*) and MN852443 (*UL54*).

Phylogenetic Analysis

Nucleotide sequences from *UL27*, *UL44* and *UL54* genes (BHV5//India/2018) were edited to yield 447, 1368 and 585 bp fragments respectively using BioEdit version 7.0. These sequences, together with the representative nucleotide sequences of BHV1 and BHV5 available in the public domain (GenBank) were subjected to multiple sequence alignments using CLUSTALW (<http://www.ebi.ac.uk/clustalw/index.html>). Phylogenetic analyses were conducted using MEGA X. [31]. In order to evaluate the evolutionary history of the strain as well as phylogenetic relationship with different lineages, a Neighbor-Joining concatermeric phylogenetic tree comprising of *UL27*, *UL44* and *UL54* genes was generated. Test of phylogeny was performed using Maximum Composite Likelihood method and the confidence interval was estimated by a bootstrap algorithm applying 1,000 iterations. Molecular phylogeny and genetic relatedness of the isolated BHV5, with the rest of the BHV1/BHV5 strains were calculated using % similarity and pairwise distances.

Results

Identification of the agent(s)

For demonstration of the etiological agent in the aborted cattle, we extracted the DNA from the vaginal swabs and subjected it for amplification of BHV1, *Campylobacter spp*, *Brucella spp*, *Leptospira spp*, *Listeria spp* and *Trichomonas vaginalis* genome, the agents which are commonly associated with abortion in cattle. Among the bacterial/parasitic agents, amplification could not be observed except for *Brucella spp* (data not shown). Amplification of a 484 nt long PCR fragment with BHV1-specific primers primarily indicated the presence of BHV1 in vaginal swabs (**Fig. 1a**). Among the three aborted cattle, two were found positive for both BHV1 and *Brucella* whereas one was positive only for BHV1 genome. The PCR product (BHV1) was subsequently gel purified and submitted for direct sequencing. Nucleotide sequence similarity using NCBI BLAST revealed a close homology with BHV5

strains, rather than with BHV1 strains. This finding was intriguing because BHV5 had never been reported from India. When we re-examined the nucleotide sequences of the primers [32] that were used to amplify *UL27* gene by PCR, the sequences were found to be conserved among BHV1 and BHV5 genome. Therefore, the primer could amplify *UL27* gene of both BHV1 and BHV5.

The virus was also subjected to differential PCR as described previously [21]. As anticipated, PCR amplification of the *UL44* gene from BHV1 (reference) and BHV5 (sample) resulted in amplification of 354 bp and 159 bp fragments respectively (**Fig. 1b**) which further confirmed that the virus detected in vaginal swab was BHV5 and not BHV1.

Virus isolation

The virus recovered from the vaginal swabs was used to infect MDBK cells, however no CPE was observed even up to 7 days post-infection. Thereafter, the infected cells were freeze-thawed twice and the resulting supernatant (called first blind passage) was used to reinfect fresh MDBK cells. In the third blind passage, the CPE characterized by cell rounding, ballooning and degeneration was observed at ~36 h post infection in virus-infected but not in mock-infected cells (**Fig. 1c**). Viral genome could be amplified in MDBK-infected cell culture supernatant at the 3rd blind passage (data not shown). The isolated virus also formed plaques in MDBK cells which were visible within 5-6 days following infection (**Fig. 1d**). The virus infected cell culture supernatant had a titre of 1.2×10^7 plaque forming unit per millilitre (PFU/ml) at the 3rd blind passage. For bulk production, the MDBK cells were infected at MOI of 0.1 of BHV5 followed by virus harvest at 48 hpi by rapid freeze-thaw method. The bulk virus had a titre of 1.4×10^7 PFU/ml. The virus was deposited in the microbial repository at NCVTC, Hisar bearing an Accession Number VTCCAVA218 and named as BHV5/*Bos taurus*-tc/India/2018/Bhilwara.

Biotyping of BHV5

Based on the restriction endonuclease pattern, BHV5 has 3 subtypes, viz., A, B and C. By employing a previously described method, we also subjected the isolated BHV5 for subtyping [22]. As per the method, multiplex PCR amplification of BHV5 *UL27* and *UL54* genes (**Fig. 2a**) and their subsequent digestion by *BstEII* produces following banding patterns: (i) Type A: *UL27* (363 bp, 171 bp) and *UL54* (420 bp, 249 bp) (ii) Type B: *UL27* (534 bp) and *UL54* (420 bp, 249 bp) (iii) Type C: *UL27* (363 bp, 171 bp) and *UL54* (649 bp). In our study, PCR amplification of *UL27* and *UL54* genes and their subsequent digestion by *BstEII* produced four DNA fragments viz; 420 bp, 363 bp, 249 bp and 171 bp (**Fig. 2b**) which were suggestive of subtype A of BHV5/India/2018/Bhilwara.

Genetic relatedness

In *UL44* (envelope glycoprotein C), the nucleotide (nt) and amino acid (aa) identities with other BHV5 strains were found to be in the range of 98.5-100% and 98.1-100% respectively and the highest identity (100%) was depicted with a Brazilian isolate (KY549446.1). The nt and aa identities in the same region with BHV1 isolates were in the range of 91.6-96.7% and 90.0-95.9% respectively. At the *UL54* region the nt identities ranged between 99.4-100% and the same were 90.4-99.5% in the partial glycoprotein B region (*UL27*) as compared to the other BHV5 isolates.

Phylogenetic analysis

Neighbour-joining phylogenetic tree comprising of *UL27*, *UL44* and *UL54* genes was constructed to ascertain the evolutionary relationship of the virus with other BHV5 isolates in the public database. The Indian isolate clustered with the Brazilian BHV5 isolates with 100% bootstrap suggesting that the virus is closely related with BHV5 isolates from Brazil (**Fig. 3**).

Analysis of recombination

To elucidate any evidence of recombination between the BHV5 isolates, by employing RDP4 programme, we also carried out recombination analysis in the *UL44* gene with the available

sequences in the public database. The default parameters available in RDP4 programme viz., RDP, GENECONV, BOOTSCAN, MAXCHI, Chimera, SISCAN and TOPAL were employed to identify the recombination breakpoints and the parental strains. However, the analysis did not reveal any evidence of recombination (data not shown).

Detection of antiviral antibodies

We also evaluated the levels of antiviral antibodies in three aborted and three apparently healthy cattle belonging to the same farm. An antibody titre of 8-128 was observed irrespective of the abortion history (Table 2) suggesting an active BHV5 infection in the herd. Serum samples of all the aborted cattle were also positive for antibodies against *Brucella* (Table 2).

Table 2: Detection of viral genome and antiviral antibodies in aborted and apparently health animals

Animal ID	Abortion history	BHV5 genome*	<i>Brucella</i> genome	Antibody titre		
				(Anti-BHV1)	(Anti-BHV5)	<i>Brucella</i> a
Mahima	Yes	(+)	(+)	64	32	(+)
Kishori	Yes	(+)	(-)	32	32	(+)
Jaya	Yes	(+)	(+)	8	8	(+)
Kavita	No	(-)	(-)	16	32	NT
Kirti	No	(-)	(-)	64	8	NT
Kapila	NO	(-)	(-)	128	128	NT

*=Vaginal swab, NT= Not tested, (+)= Positive, (-)= Negative

Discussion

BHV5 distribution is restricted to South American countries, particularly Argentina [9], Uruguay [10] and Brazil [11]. Only a few cases of this disease have been reported from other countries [12,13,15,16]. BHV5 has never been reported from India. Clinical findings, detection of antiviral antibodies, virus isolation, BHV1/BHV5 differential PCR, biotyping and sequence and phylogenetic analysis of *UL27*, *UL44* and *UL54* genes confirmed the association of BHV5 subtype A in the aborted cattle. To the best of our knowledge this is the first report on the presence of BHV5 infection in India.

DNA extracted from vaginal swabs of aborted cattle showed the presence of BHV1 (three aborted cattle) and *Brucella* (2 aborted cattle). Other agents such as *Campylobacter* spp, *Listeria* spp, *Leptospira* spp, *Trichomonas vaginalis* were not detected. Rapid generation of CPE (within 36 hrs) in MDBK cells, a characteristic of BHV1 further indicated association of BHV1 in aborted cattle. However, nucleotide sequences revealed a close homology with the BHV5 strains, rather than with the BHV1 strains. When the corresponding nucleotide sequences of the primers [32] used to amplify *UL27* gene were re-examined, they were identical in BHV1 and BHV5 genomes. Therefore, the primer could amplify *UL27* gene of both BHV1 and BHV5 strains.

In order to further confirm, the isolated virus (BHV5//India/2018/Bhilwara) and a reference BHV1 control were subjected to differential PCR as described previously (Claus et al., 2005). As anticipated, PCR amplification of the *UL44* gene from BHV1 and BHV5 resulted in amplification of 354 bp and 159 bp fragments, respectively, which further confirmed the identity of the virus as BHV5, not BHV1.

Based on the restriction endonuclease patterns [22,30], BHV5 has 3 subtypes, viz., A, B and C. Type strains for subtypes A, B and non-A-non-B, are the Australian strain N569, the Argentinean strain A663 and Brazilian strains, respectively. The banding patterns generated

following PCR amplification of *UL54* and *UL27* genes (BHV5//India/2018/Bhilwara) and their subsequent digestion by *BstEII* were clearly suggestive of BHV5 subtype A.

BHV5 infection in cattle usually causes meningoencephalitis [3], although few reports also suggest the involvement of the reproductive tract [4]. Besides demonstration of the virus (BHV5) and BHV5-specific antibodies in the aborted animals, no obvious neurological signs could be recorded in any of the aborted cattle. There are reports suggesting interspecific recombination between BHV1 and BHV5 [33]. However our analysis did not reveal any evidence of recombination. Besides, we could not detect evidence of BHV1 infection also in the farm. Although reproducing clinical BHV5 disease under experimental conditions is a tedious task, some laboratories have developed a rabbit model of encephalitis [34-38]. Although the isolated virus in our study was ~99% identical with the Brazilian BHV5 strains, its complete genetic characterization (whole genome sequencing) as well as its ability to produce encephalitis in natural host and/or in rabbits needs to be elucidated which is beyond the scope of this manuscript. Likewise, precise role of the isolated virus (BHV5/India/2018/Bhilwara) and/or other infectious agents (coinfection) in inducing abortion in the cattle farm needs to be determined.

Besides demonstration of the etiological agent(s), serum samples were also found positive for both anti-BHV1/5 and anti-*Brucella* antibodies suggesting an active BHV1 and *Brucella* coinfection in the herd. Some serological evidences have shown BHV1 and *Brucella* coinfection [39-42], however, demonstration of agent (BHV1/5 and *Brucella*) has been a rare event. It is a matter of further study whether *Brucella* and BHV5 coinfection have synergistic or antagonistic effect on disease severity [43].

BHV1 vaccine induces cross-protection against BHV5 disease in cattle [7]. Naturally occurring or vaccine-induced anti-BHV1 antibodies are believed to reduce the occurrence of BHV5-associated disease in BHV1 endemic areas [44]. However, with reasons precisely

unknown, this does not apply to the epidemiology and transmission of BHV5 in South America. Even though Argentina and Brazil have a high percentage of BHV1 seropositive cattle (24.8–84.1% and 19–85%, respectively) [reviewed in reference [3]], both countries have reported several cases of BHV5 associated meningoencephalitis. Furthermore, serological tests which can differentiate anti-BHV1 and anti-BHV5 antibodies are not available. Thus, the actual prevalence of BHV5 infection and hence economic significance remains unknown. Furthermore, the phenomenon of viral interference between BHV1 and BHV5 needs to be studied.

Like Europe and USA, since India has no specific programme for the detection and identification of BHV5 infected animals, it might have been overlooked despite its presence. The concerned farmer always practiced natural service and never performed artificial insemination for breeding purposes. However, the bulls were procured from nearby state (Surendranagar, Gujrat). India import semen from several other countries including Brazil, therefore the possibility of introduction of BHV5 via semen from Brazil and/or other countries cannot be ruled out.

Very few studies have been undertaken on the development of BHV5 vaccine, firstly because of its limited geographical distribution and secondly reproducing the clinical BHV5 disease under experimental conditions is difficult [3,45]. Countries with frequent BHV5 outbreaks along with high prevalence of BHV1 have successfully employed BHV1 vaccines for protection against BHV5 infection [7,8]. However, the levels of anti-BHV5 antibodies induced by BHV1 vaccine is usually low and of shorter duration [46]. Therefore, each BHV1 vaccine should be carefully tested for potential cross-protection against BHV5.

To conclude, we provide a strong evidence of BHV5 infection in Indian cattle for the first time. The isolated virus would be useful for developing diagnostic, prophylactic and

therapeutic agents to combat BHV5 disease in India. The finding may necessitate inclusion of BHV5 test protocol in testing of semen for sexually transmitted diseases.

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Author contribution

N-Ku and R-T designed the experiments. N-Ku, Y-C, N-Kh, R-K and H-K performed the experiments. N-Ku, S-B, R-T and B-NT wrote the manuscript.

References

1. Engels M, Giuliani C, Wild P, Beck TM, Loepfe E, et al. (1986) The genome of bovine herpesvirus 1 (BHV-1) strains exhibiting a neuropathogenic potential compared to known BHV-1 strains by restriction site mapping and cross-hybridization. *Virus Res* 6: 57-73.
2. Schudel AA, Carrillo BJ, Wyler R, Metzler AE (1986) Infections of calves with antigenic variants of bovine herpesvirus 1 (BHV-1) and neurological disease. *Zentralbl Veterinarmed B* 33: 303-310.
3. Del Medico Zajac MP, Ladelfa MF, Kotsias F, Muylkens B, Thiry J, et al. (2010) Biology of bovine herpesvirus 5. *Vet J* 184: 138-145.
4. Favier PA, Marin MS, Perez SE (2012) Role of bovine herpesvirus type 5 (BoHV-5) in diseases of cattle. Recent findings on BoHV-5 association with genital disease. *Open Vet J* 2: 46-53.

5. Studdert MJ (1989) Bovine encephalitis herpesvirus. *Vet Rec* 125: 584.
6. Roizmann B, Desrosiers RC, Fleckenstein B, Lopez C, Minson AC, et al. (1992) The family Herpesviridae: an update. The Herpesvirus Study Group of the International Committee on Taxonomy of Viruses. *Arch Virol* 123: 425-449.
7. Del Medico Zajac MP, Puntel M, Zamorano PI, Sadir AM, Romera SA (2006) BHV-1 vaccine induces cross-protection against BHV-5 disease in cattle. *Res Vet Sci* 81: 327-334.
8. d'Offay JM, Ely RW, Baldwin CA, Whitenack DL, Stair EL, et al. (1995) Diagnosis of encephalitic bovine herpesvirus type 5 (BHV-5) infection in cattle: virus isolation and immunohistochemical detection of antigen in formalin-fixed bovine brain tissues. *J Vet Diagn Invest* 7: 247-251.
9. Carrillo BJ, Ambrogi A, Schudel AA, Vazquez M, Dahme E, et al. (1983) Meningoencephalitis caused by IBR virus in calves in Argentina. *Zentralbl Veterinarmed B* 30: 327-332.
10. Guarino H, Nunez A, Repiso MV, Gil A, Dargatz DA (2008) Prevalence of serum antibodies to bovine herpesvirus-1 and bovine viral diarrhea virus in beef cattle in Uruguay. *Prev Vet Med* 85: 34-40.
11. Campos FS, Franco AC, Hubner SO, Oliveira MT, Silva AD, et al. (2009) High prevalence of co-infections with bovine herpesvirus 1 and 5 found in cattle in southern Brazil. *Vet Microbiol* 139: 67-73.
12. Kessell A, Finnie J, Windsor P (2011) Neurological diseases of ruminant livestock in Australia. IV: viral infections. *Aust Vet J* 89: 331-337.
13. Bartha A, Hajdu G, Aldasy P, Paczolay G (1969) Occurrence of encephalitis caused by infectious bovine rhinotracheitis virus in calves in hungary. *Acta Vet Acad Sci Hung* 19: 145-151.

14. Sharifzadeh A, Namazi MJ, Mokhtari-Farsani A, Doosti A (2015) Bovine herpesvirus type 5 in semen samples from bulls in Iran. Arch Virol 160: 235-239.
15. Gough A, James D (1975) Isolation of IBR virus from a heifer with meningoencephalitis. Can Vet J 16: 313-314.
16. Barenfus M, Delliquadri CA, McIntyre RW, Schroeder RJ (1963) Isolation of Infectious Bovine Rhinotracheitis Virus from Calves with Meningoencephalitis. J Am Vet Med Assoc 143: 725-728.
17. Kumar N, Barua S, Riyesh T, Chaubey KK, Rawat KD, et al. (2016) Complexities in Isolation and Purification of Multiple Viruses from Mixed Viral Infections: Viral Interference, Persistence and Exclusion. PLoS One 11: e0156110.
18. Khandelwal N, Chander Y, Rawat KD, Riyesh T, Nishanth C, et al. (2017) Emetine inhibits replication of RNA and DNA viruses without generating drug-resistant virus variants. Antiviral Res 144: 196-204.
19. Kumar N, Wadhwa A, Chaubey KK, Singh SV, Gupta S, et al. (2014) Isolation and phylogenetic analysis of an orf virus from sheep in Makhdoom, India. Virus Genes 48: 312-319.
20. Rocha MA, Barbosa EF, Guedes RM, Lage AP, Leite RC, et al. (1999) Detection of BHV-1 in a naturally infected bovine fetus by a nested PCR assay. Vet Res Commun 23: 133-141.
21. Claus MP, Alfieri AF, Folgueras-Flatschart AV, Wosiacki SR, Medici KC, et al. (2005) Rapid detection and differentiation of bovine herpesvirus 1 and 5 glycoprotein C gene in clinical specimens by multiplex-PCR. J Virol Methods 128: 183-188.
22. Maidana SS, Morano CD, Cianfrini D, Campos FS, Roehe PM, et al. (2013) Multiplex PCR followed by restriction length polymorphism analysis for the subtyping of bovine herpesvirus 5 isolates. BMC Vet Res 9: 111.

23. Leal-Klevezas DS, Martinez-Vazquez IO, Lopez-Merino A, Martinez-Soriano JP (1995) Single-step PCR for detection of *Brucella* spp. from blood and milk of infected animals. *J Clin Microbiol* 33: 3087-3090.
24. Nayak DN, Savalia CV, Kalyani IH, Kumar R, Kshirsagar DP (2015) Isolation, identification, and characterization of *Listeria* spp. from various animal origin foods. *Vet World* 8: 695-701.
25. Merien F, Amouriaux P, Perolat P, Baranton G, Saint Girons I (1992) Polymerase chain reaction for detection of *Leptospira* spp. in clinical samples. *J Clin Microbiol* 30: 2219-2224.
26. Linton D, Lawson AJ, Owen RJ, Stanley J (1997) PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. *J Clin Microbiol* 35: 2568-2572.
27. Madico G, Quinn TC, Rompalo A, McKee KT, Jr., Gaydos CA (1998) Diagnosis of *Trichomonas vaginalis* infection by PCR using vaginal swab samples. *J Clin Microbiol* 36: 3205-3210.
28. Kumar R, Khandelwal N, Chander Y, Riyesh T, Tripathi BN, et al. (2018) MNK1 inhibitor as an antiviral agent suppresses buffalopox virus protein synthesis. *Antiviral Res* 160: 126-136.
29. Kumar N, Khandelwal N, Kumar R, Chander Y, Rawat KD, et al. (2019) Inhibitor of Sarco/Endoplasmic Reticulum Calcium-ATPase Impairs Multiple Steps of Paramyxovirus Replication. *Front Microbiol* 10: 209.
30. D'Arce RC, Almeida RS, Silva TC, Franco AC, Spilki F, et al. (2002) Restriction endonuclease and monoclonal antibody analysis of Brazilian isolates of bovine herpesviruses types 1 and 5. *Vet Microbiol* 88: 315-324.

31. Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol Biol Evol* 35: 1547-1549.
32. Vilcek S, Nettleton PF, Herring JA, Herring AJ (1994) Rapid detection of bovine herpesvirus 1 (BHV 1) using the polymerase chain reaction. *Vet Microbiol* 42: 53-64.
33. Meurens F, Keil GM, Muylkens B, Gogev S, Schynts F, et al. (2004) Interspecific recombination between two ruminant alphaherpesviruses, bovine herpesviruses 1 and 5. *J Virol* 78: 9828-9836.
34. Chowdhury SI, Mahmood S, Simon J, Al-Mubarak A, Zhou Y (2006) The Us9 gene of bovine herpesvirus 1 (BHV-1) effectively complements a Us9-null strain of BHV-5 for anterograde transport, neurovirulence, and neuroinvasiveness in a rabbit model. *J Virol* 80: 4396-4405.
35. Chowdhury SI, Lee BJ, Onderci M, Weiss ML, Mosier D (2000) Neurovirulence of glycoprotein C(gC)-deleted bovine herpesvirus type-5 (BHV-5) and BHV-5 expressing BHV-1 gC in a rabbit seizure model. *J Neurovirol* 6: 284-295.
36. Chowdhury SI, Lee BJ, Ozkul A, Weiss ML (2000) Bovine herpesvirus 5 glycoprotein E is important for neuroinvasiveness and neurovirulence in the olfactory pathway of the rabbit. *J Virol* 74: 2094-2106.
37. Chowdhury SI, Lee BJ, Mosier D, Sur JH, Osorio FA, et al. (1997) Neuropathology of bovine herpesvirus type 5 (BHV-5) meningo-encephalitis in a rabbit seizure model. *J Comp Pathol* 117: 295-310.
38. Machado GF, Bernardi F, Hosomi FY, Peiro JR, Weiblen R, et al. (2013) Bovine herpesvirus-5 infection in a rabbit experimental model: immunohistochemical study of the cellular response in the CNS. *Microb Pathog* 57: 10-16.

39. Aslan ME, Azkur AK, Gazyagci S (2015) Epidemiology and genetic characterization of BVDV, BHV-1, BHV-4, BHV-5 and Brucella spp. infections in cattle in Turkey. J Vet Med Sci 77: 1371-1377.
40. Morris MJ, Sookhoo J, Blake L, Brown Jordan A, John J, et al. (2018) Serosurvey for Infectious Agents Associated with Subfertility and Abortion in Dairy Cattle in Trinidad and Tobago, West Indies. Vet Sci 5.
41. Vidal S, Kegler K, Greub G, Aeby S, Borel N, et al. (2017) Neglected zoonotic agents in cattle abortion: tackling the difficult to grow bacteria. BMC Vet Res 13: 373.
42. Konnai S, Mingala CN, Sato M, Abes NS, Venturina FA, et al. (2008) A survey of abortifacient infectious agents in livestock in Luzon, the Philippines, with emphasis on the situation in a cattle herd with abortion problems. Acta Trop 105: 269-273.
43. Kumar N, Sharma S, Barua S, Tripathi BN, Rouse BT (2018) Virological and Immunological Outcomes of Coinfections. Clin Microbiol Rev 31.
44. d'Offay JM, Mock RE, Fulton RW (1993) Isolation and characterization of encephalitic bovine herpesvirus type 1 isolates from cattle in North America. Am J Vet Res 54: 534-539.
45. Moran PE, Perez SE, Odeon AC, Verna AE (2015) [Bovine herpesvirus 4 (BoHV-4): general aspects of the biology and status in Argentina]. Rev Argent Microbiol 47: 155-166.
46. Petzhold SA RP, Prado JAP, Teixeira JC, Wald VB, Esteves PA, ESTEVES1; Spilki FR, Roehe PM (2001) Neutralizing antibodies to bovine herpesviruses types 1 (BHV-1) and 5 (BHV-5) induced by an experimental, oil-adjuvanted, BHV-1 vaccine. Braz J Vet Res Anim Sci 38: 184-187.

Figure legends

Fig. 1. Isolation and identification of the agent. (a) Amplification of BHV1 genome in

vaginal swab. Virus was recovered from the vaginal swabs in DMEM followed by DNA

extraction and PCR to amplify *UL27* gene of BHV1. **(b) BHV1/BHV5 differential PCR.**

PCR was carried out to amplify *UL44* as per the method described by Claus *et al.* PCR

amplification of *UL44* from BHV1 (reference) and BHV5 (sample) resulted in amplification

of 354 bp and 159 bp fragments respectively. **(c). Virus isolation in MDBK cells.** 500 µl of

the virus recovered from the vaginal swab was used to infect MDBK cells. Cytopathic effect

was observed at 3rd blind passage. Virus-infected and mock-infected cells are shown. **(d)**

Plaque assay. Confluent monolayers of MDBK cells were infected with 10-fold serial

dilutions of the virus for 1 h at 37°C followed by replaced the medium with an agar-overlay.

The plaques were visible at 5-6 days after infection. The agar-overlay was removed, and the

plaques were stained by 1% crystal violet. The viral titers were measured in plaque forming

unit/ml (pfu/ml).

Fig. 2. BHV5 subtyping: (a) Amplification of UL27 and UL54 genes of BHV5 by multiplex

PCR resulted in amplification of 534 bp and 649 bp fragments respectively. **(b) Digestion of**

PCR products (multiplex-*UL27* and *UL54* genes) by *BstEII* resulted in amplification of 420

bp, 363 bp, 249 bp and 171 bp fragments.

Fig. 3. Phylogenetic analysis: Nucleotide sequences from *UL27*, *UL44* and *UL54* genes

(BHV5//India/2018) were edited to 447, 1368 and 585 bp fragments respectively, using

BioEdit version 7.0. These sequences, together with the representative nucleotide sequences

of BHV1 and BHV5 available in the public domain (GenBank) were subjected for multiple

sequence alignments Phylogenetic analyses were conducted using MEGA X. To evaluate the

evolutionary history of the strain as well as the phylogenetic relationship with different lineages, a concatenomic Neighbor-Joining method tree was generated. Test of phylogeny was performed using Maximum Composite Likelihood method and the confidence intervals were estimated by a bootstrap algorithm applying 1,000 iterations. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Fig. 1

Figure 1

Fig. 2

Figure 2

Figure 3

Figure 3