Genome sequence and experimental infection of calves with bovine gammaherpesvirus 4 (BoHV-4)

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Abstract

Bovine gammaherpesvirus 4 (BoHV-4) is ubiquitous in cattle worldwide, and it has been detected in animals exhibiting broad clinical presentations. The virus has been detected in the United States since the 1970s; however, its clinical relevance remains unknown. Here, we determined the complete genome sequences of two contemporary BoHV-4 isolates obtained from respiratory (SD16-38) or reproductive (SD16-49) tract specimens and assessed clinical, virological, and pathological outcomes upon intranasal (IN) inoculation of calves with the respiratory BoHV-4 isolate SD16-38. A slight and transient increase in body temperature was observed in BoHV-4-inoculated calves. Additionally, transient viremia and virus shedding in nasal secretions were observed in all inoculated calves. BoHV-4 DNA was detected by nested PCR in the tonsil and regional lymph nodes (LNs) of calves euthanized on day 5 post-inoculation (pi) and in the lungs of calves euthanized on day 10 pi. Calves euthanized on day 35 pi harbored BoHV-4 DNA in the respiratory tract (turbinates, trachea, lungs), regional lymphoid tissues, and trigeminal ganglia. Interestingly, in situ hybridization revealed the presence of BoHV-4 DNA in nerve bundles surrounding the trigeminal ganglia and retropharyngeal lymph nodes (day 35 pi). No histological changes were observed in the respiratory tract (turbinate, trachea, and lung), lymphoid tissues (tonsil, LNs, thymus, and spleen), or central nervous tissues (olfactory bulb and trigeminal ganglia) sampled throughout the animal studies (days 5, 10, and 35 pi). This study contributes to the understanding of the infection dynamics and tissue distribution of BoHV-4 following IN infection in calves. These results suggest that BoHV-4 SD16-38 used in our study has low pathogenicity in calves upon intranasal inoculation.

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Introduction

Bovine gammaherpesvirus 4 (BoHV-4) is widespread in cattle worldwide, and it has been isolated from healthy animals and from animals with a variety of clinical signs, including respiratory or reproductive disorders [1–5]. The role of BoHV-4 in overt disease, however, remains unknown. The virus has a broad geographical distribution and has been detected in cattle in Europe, America, Asia, and Africa [6–10]. Originally, BoHV-4 was classified in the subfamily Betaherpesvirinae, genus Cytomegalovirus, based on the formation of high-density inclusion bodies and induction of giant cells in vitro. However, genome structure analysis [11] and the discovery of the presence of a thymidine kinase gene [12] led to its re-classification in the genus Rhadinovirus, subfamily Gammaherpesvirinae [13]. The BoHV-4 genome consists of a double-stranded DNA molecule with ~108,873 bp and an overall G+C content of 41.4%, containing at least 79 open reading frames, with 17 of them being unique to BoHV-4 [14].

The virus was first isolated in 1963 in Hungary from calves exhibiting keratoconjunctivitis and respiratory distress [6]. In 1971, BoHV-4 was reported in the United States following isolation from tissues of a bovine with respiratory disease [8]. Based on genomic restriction patterns, two BoHV-4 groups were initially recognized and named after the prototype virus strains [15]. The European group was named "M ovar 33/63-like viruses", whereas the American group was referred to as "DN599-like viruses" [15]. Viruses of these two groups are not restricted geographically [11], and given their genetic differences, the European and American groups have been suggested to belong to genotype 1 and 2, respectively [16]. The biological relevance of the genomic differences observed in the two BoHV-4 genotypes, however, remains unknown. While members of the family Bovidae are thought to be the natural hosts of BoHV-4, it is hypothesized that the virus originally circulated among African bison and might have "spilled over" to cattle [17]. In cattle, BoHV-4 has been isolated from healthy animals as well as from animals with a variety of clinical conditions, including abortion, metritis, vulvovaginitis, mastitis, pneumonia, keratoconjunctivitis, and dermatitis [1, 6–8, 18–22]. The role of the virus in these clinical manifestations, however, is largely unknown. The tropism of BoHV-4 for cells of the monocyte/macrophage lineage during lytic and persistent/latent infection [23] may explain – at least in part – the frequent isolation of the virus from diverse tissues and inflammatory conditions. Experimental inoculation of BoHV-4 in cattle through different inoculation routes has demonstrated that the virus primarily replicates in epithelial cells in the nasal mucosa, followed by viremia associated with peripheral blood mononuclear cells (PBMCs) [20, 24–26]. It has been suggested that BoHV-4 may establish latent infection in lymphoid and neuronal tissues [20, 25–27].

In the present study, we determined the complete genome sequences of two contemporary BoHV-4 isolates obtained from respiratory and reproductive specimens and assessed the clinical, virological, and pathological outcomes of infection upon intranasal inoculation of calves with the respiratory BoHV-4 isolate SD16-38.

Materials and methods

Viruses and cells

The isolates SD16-38 and SD16-49 were obtained in 2016 at the Animal Disease Research and Diagnostic Laboratory at South Dakota State University (ADRDL-SDSU) using bovine turbinate (BT) cells (ATCC; CRL-1390). BoHV-4 SD16-49 was isolated from a cow with a reproductive disorder, and isolate SD16-38 was recovered from lung tissue from a calf with respiratory disease. The isolate SD16-38 was amplified and titrated in BT cells, which were tested and found to be free of non-cytopathic bovine viral diarrhea virus (BVDV). Cells were cultured at 37 °C with 5% CO₂ in minimum essential medium (MEM) (Corning, Corning, NY) supplemented with 10% fetal bovine serum (FBS; VWR, Radnor, PA), penicillin (100 IU/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml) (Corning, Corning, NY). Low-passage viral stocks (P.4) for both SD16-38 and SD16-49 isolates were used in this study.

Whole genome sequence and characterization

Supernatants of BoHV-4-inoculated BT cell monolayers (SD16-38 and SD16-49; P.4) were subjected to high-throughput sequencing using an Illumina MiSeq sequencing platform. DNA libraries were prepared using a Nextera XT DNA Library Kit (Illumina) following the manufacturer’s protocol. The DNA library was quantitated using a Qubit dsDNA Assay Kit (Life Technologies). Library DNA (4 nM) was loaded into a MiSeq Nano Flow Cell (300 cycles, Illumina) and sequenced using an Illumina MiSeq sequencing platform (Illumina). Three individual rounds of sequencing were performed, and the results were combined to generate a consensus sequence for both BoHV-4 isolates. The BoHV-4 genome sequences were assembled using Ray [28], and the repeats were resolved using Celera [29] and Cap3 [30] software. Open reading frames (ORFs) in BoHV-4 isolates SD16-38 and SD16-49 were identified and annotated using Geneious Prime software based on the genomic sequences of BoHV-4 strains 66-p-377 and FMV09 (GenBank accession numbers AF318573 and KC999113.1).
Animal study design

The pathogenicity and infection dynamics of BoHV-4 isolate SD16-38 was evaluated in calves. For this, eighteen 3-week-old colostrum-deprived Holstein bull calves negative for BoHV-4 neutralizing antibodies were separated into three groups [31]. After seven days of acclimation, 10 calves were inoculated with a suspension of BoHV-4 isolate SD16-38 (10^6.0 TCID50/mL) via IN instillation (5 ml in each nostril). Four calves were mock-inoculated with MEM (5 ml in each nostril) and kept as controls, and the last four calves served as non-inoculated contact animals that were allowed to come in with the inoculated animals starting at day 3 pi to evaluate potential virus transmission. Nasal swabs and blood samples were collected on days -1, 0, 3, 5, 7, and 10 pi. Two control calves and four inoculated calves were euthanized on days 5 pi and 10 pi. The four contact calves were euthanized on day 10 pi. The two remaining inoculated calves were euthanized on day 35 pi. Additional serum sampling for neutralizing antibody titration was performed on day 35 pi for the two inoculated calves. During the course of the study, animals were monitored daily and scored for clinical signs using the Calf Health Scorer system developed at the University of Wisconsin School of Veterinary Medicine.

Body temperature was monitored constantly using ruminal temperature sensors implanted for the study, animals were monitored daily and scored for clinical signs using the Calf Health Scorer system developed at the University of Wisconsin School of Veterinary Medicine. Body temperature was monitored constantly using ruminal probes as described previously [31]. Euthanasia was performed using a pentobarbital sodium injection (Fatal Plus, Vortech Pharmaceutical Ltd., Dearborn, MI). The animals were handled in accordance with the Animal Welfare Act Amendments (7 U.S. Code §2131 to §2156), and all procedures were approved by the Institutional Animal Care and Use Committee of the National Animal Disease Center (ARS-2016-572).

Sample collection and processing

Blood samples were collected by jugular venipuncture with Vacuette tubes containing clot activator (for serum), heparin (for PBMCs), or ethylenediaminetetraacetic (for complete blood cell count; CBC) (Greiner Bio-One, Monroe, NC). Serum samples were aliquoted and stored at −20 °C until tested. Blood samples collected with heparin were centrifuged at 1200 × g for 10 min, and the buffy coat was transferred to a cryotube and stored at −80 °C until use. Tissues collected at necropsy included nasal turbinate, thymus, tonsil, lung, spleen, olfactory bulb, trigeminal ganglia, and lymph nodes (mandibular, retropharyngeal, and tracheobronchial). Tissues were fixed by immersion in 10% neutral buffered formalin for RNAscope testing and placed in bags and frozen for DNA extraction and PCR testing. Tissues were stored at −80 °C until use.

Viral DNA extraction and nested PCR

Total DNA was extracted from serum, buffy coats, and nasal swab samples using QIAamp® DNA (QIAGEN) according to the manufacturer’s instructions. Total DNA was extracted from tissues as follows: approximately 1 g of tissue was minced with a sterile scalpel blade and mixed with 9 ml of MEM (1:10 w/v). Samples were homogenized with a Stomacher and centrifuged at 1200 × g for 10 min. DNA was extracted from tissue homogenates using QIAamp® DNA (QIAGEN) according to the manufacturer’s instructions. BoHV-4 DNA was detected using a previously described nPCR assay [24] with minor modifications. Briefly, nPCR targeting the BoHV-4 thymidine kinase region employed the following primers: 5’-GTTTGGCGCTCCTGATGG TAGC-3’ (primer 1), 5’-ATGTATGCCCCAATCTTATAA TATGACCAG-3’ (primer 2), 5’-TTGATAGTGCTTGTGT TGGGATGTGG-3’ (primer 3), and 5’-CAGTCGCCTGG TGGAAATGCA-3’ (primer 4). Primers 1 and 2 flanked a 567-bp fragment, and the internal primers (primers 3 and 4) amplified a 260-bp product. Both PCR amplifications were performed following the same protocol, using 50-μl reactions containing 25 μl of Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs), 2.5 μl of each primer (final concentration, 0.5 μM), 19 μl of nuclelease-free water (Thermo-Fisher Scientific), and 1 μl of purified nucleic acid as template (~10 ng of total DNA per reaction). The PCR conditions consisted of five initial amplification cycles at 94 °C for 45 s, 56 °C for 1 min, and 72 °C for 1.5 min. Subsequently, an additional 25 cycles of 94 °C for 45 s, 51 °C for 1 min, and 72 °C for 1.5 min were performed, followed by 72 °C for 5 min. PCR amplicons were analyzed by 1% agarose gel electrophoresis. Positive (viral culture) and negative (no template) controls were included in each reaction.

Virus isolation and quantification

Virus isolation in cell cultures was attempted from serum, buffy coats, nasal swabs, and tissue samples using BT cells cultured in 24-well plates. Nasal swabs were placed in microtubes with 500 μl of MEM and vortexed for at least 30 s, and 150 μl of this suspension was used to inoculate the cells in each well. For buffy coats and serum, 150 μl of suspension was added to each well, and the virus was allowed to adsorb to the cells for 1 h. Tissues were processed as described above, and 150 μl of the tissue homogenate was added to each well. Cells were washed twice with MEM, and 500 μl of replacement medium supplemented with 10% FBS was added to each well. Three passages were performed in 70–80% confluent cell monolayers. Plates were incubated at 37°C with 5% CO₂ and monitored daily for 6 days.
**Virus neutralization assays**

Virus neutralization (VN) assays were performed with serum samples from all animals, collected on days 5, 10, and/or 35 pi. Briefly, serum dilutions from 1:2 to 1:512 were incubated with 200 TCID50 of the homologous virus (isolate SD16-38) for 90 min at 37 °C, followed by addition of a suspension of BT cells and incubation for 7 days at 37 °C in 5% CO2. The cells were examined under a light microscope for the presence of a cytopathic effect. Neutralizing antibody titers were determined as the reciprocal of the highest serum dilution capable of completely inhibiting BoHV-4 replication.

**RNAscope® in situ hybridization**

An RNAscope® in situ hybridization assay was used to investigate the presence of viral nucleic acid in tissues collected from the animals necropsied on day 35 pi. Tissue sections were fixed by immersion in 10% neutral buffered formalin for 24-36 h, kept in 70% ethyl alcohol until processing by standard paraffin-embedding techniques, and cut into 5-μm sections for in situ labeling of viral nucleic acid. The sequence of BoHV-4 isolate SD16-38 was used to design gene-specific oligonucleotide target probes as per the manufacturer’s recommendations. Labeling of viral nucleic acid using proprietary probes designed specifically for identification of BoHV-4 (Advanced Cell Diagnostics, Hayward, CA, USA) was performed according to manufacturer’s instructions for RNAscope® 2.0. Positive and negative control probes were used for verification of the specificity of the assay. The positive control probe consisted of a proprietary probe for *Bos taurus* ubiquitin C (UBC; Cat # 312038), while the negative control probe targeted *Bacillus subtilis* dapB (Cat # 312038).

Upon completion of the RNAscope® 2.0 assay and drying for approximately 15 min, coverslips were placed on the slides using mounting medium (EcoMount, Biocare Medical, Concord, CA, USA). The slides were scanned at 40X magnification, and images were digitized using an Aperio ScanScope XT workstation (Aperio Technology, Inc., Vista, CA, USA). Digitized images were visualized using image analysis software (HALO™, Indica Labs, Inc., Corrales, NM) to describe BoHV-4 labeling patterns and compare them to histological observations.

**Results**

**Sequencing and genome characterization**

The complete genome sequences of both BoHV-4 isolates (SD16-38 and SD16-49) were determined after amplification in BT cells. Low-passage viral stocks (p. 4) were used for sequencing. Genome sequencing of isolate SD16-38 (GenBank accession number MN551083) followed by BLASTn analysis (National Library of Medicine) of the 108,669-nt-long unique region (LUR) revealed over 99.8% nucleotide sequence identity to isolate 66-p-377 (AF318573), a member of BoHV-4 genotype 1. The coding regions of conserved genes typically employed for BoHV-4 classification, including the DNA polymerase (DNA pol) and thymidine kinase (TK) genes, displayed 99.7% and 100% nucleotide (nt) sequence identity, respectively, between SD16-38 and 66-p-377. The glycoproteins gB, gH, and gM of BoHV-4 SD16-38 and 66-p-377 were 100% identical, whereas the gL sequences were 99.3% identical. The SD16-49 genome (MN551084) shares the most sequence similarity with another member of BoHV-4 genotype 1 (isolate FMV09, KC999113.1), with over 99.9% identity in the LUR, which is 108,478 nt in length. The DNA pol, TK, gB, gH, and gM coding regions of SD16-49 and FMV09 were 100% identical, whereas the gL sequences were 99.5% identical. The gB, gH, gL, and gM coding regions of SD16-38 and SD16-49 were 95.5%, 96.1%, 98.6%, and 99.5% identical, respectively, whereas the coding regions for the DNA pol and TK were 98.8% and 99.6% identical, respectively.

**Clinical and virological findings following intranasal inoculation with BoHV-4**

No overt respiratory signs were observed in the 10 colostrum-deprived 5-week-old calves following IN inoculation with BoHV-4 isolate SD16-38. A slight transient increase in body temperature was observed at days 8 and 9 pi (Fig. 1A). In addition, inoculated animals showed a slight decrease in their lymphocyte counts between days 5 and 7 pi (Fig. 1B). Both contact and control calves remained healthy throughout the observation period.

BoHV-4 DNA was detected by nPCR in nasal secretions from all BoHV-4-inoculated calves on day 3 pi and in the majority of nasal swabs collected on days 5 and 7 pi (Table 1). Attempts to isolate the virus from nasal swabs, serum, and buffy coats were unsuccessful. Nonetheless, viral DNA was detected by nPCR in the blood (buffy coat, serum) of two contact calves (days 3 and 10 pi), suggesting virus transmission from the inoculated calves. Viral DNA was detected transiently in serum and buffy coats from most (8/10) of the inoculated animals between days 3 and 10 pi. These results indicated the presence of virus in the respiratory tract, transient viremia, and short-term shedding of virus in nasal secretions.

The use of nPCR to detect BoHV-4 in tissues collected during necropsy indicated the presence of viral DNA in at least one tissue from all but one calf, necropsied at 5 dpi (#4096) (Table 2). No viral DNA was detected in tissues from animals in the contact or control groups. Three animals euthanized...
on day 5 pi were positive for viral DNA in the tonsils and/or mandibular lymph node. Viral DNA was detected in the lungs, respiratory tract lymph nodes, and/or spleen of calves euthanized at day 10 pi. No infectious virus was isolated from PCR-positive tissues collected throughout our study. Interestingly, in both calves euthanized at day 35 pi, most of the tissues were positive. The use of RNAscope® in situ hybridization supported the nPCR findings, showing the presence of viral DNA in both neuronal and lymphoid tissues of animals euthanized at day 35 pi (Fig. 2A and B). Low levels of neutralizing
antibodies were detected in sera of two calves at day 35 pi (Table 1).

**Discussion**

In the present study, we characterized the genomic features of two contemporary BoHV-4 isolates and assessed the infection outcome following IN inoculation of calves.
with the respiratory isolate (SD16-38). The BoHV-4 isolate SD16-38 obtained from a case of respiratory disease showed the most similarity to the BoHV-4 genotype 1 isolate 66-p-377 (AF318573), which was identified in the 1960s [32]. The reproductive BoHV-4 isolate SD16-49 showed the most similarity to BoHV-4 isolate FMV09 (KC999113.1), which was obtained recently (2009) from cattle with respiratory disease in Canada [7]. Detection of BoHV-4 genotype 1 in the USA supports the notion that the so-called European BoHV-4 isolates are not geographically restricted to the cattle population in Europe. In fact, other members of BoHV-4 genotype 1 have been already detected in the USA, Canada, and Argentina [7, 33, 34].

Results from recent animal studies suggest that the clinical consequences of BoHV-4 infection likely depend upon viral, host, and environmental factors. A study with five BoHV-4 strains verified that only one strain produced respiratory disease in calves [26]. Similar to previous studies, no overt respiratory and/or systemic signs could be reproduced upon IN inoculation of calves [25, 35]. These findings seem to contrast with detection and isolation of the virus from cattle with respiratory disease [6–8]. Nonetheless, the role and contribution of BoHV-4 to respiratory disease in cattle is not clear. It is conceivable that, like other bovine respiratory infectious agents, BoHV-4 may act as a cofactor in bovine respiratory disease syndrome [36]. The ubiquitous nature of BoHV-4, its frequent isolation from healthy animals, its presence in the cell fraction of bulk milk, and its association with inflammatory conditions of the respiratory and reproductive tract, combined with its ability to infect circulating monocytes, however, also suggest that detection of the virus in such a broad array of clinical presentations might be incidental. Results from our study, in which calves were inoculated with high viral titers via the IN route and did not develop overt respiratory disease, suggest that BoHV-4 may not be a primary pathogen of respiratory disease in cattle.

Nonetheless, our results show that BoHV-4 efficiently replicated in the nasal mucosa and was shed transiently by inoculated animals. Additionally, transient and intermittent viremia was observed. Viremia was detected in eight out of 10 animals between days 3 and 10 pi and was typically transient in all of them. A transient increase in body temperature was the only clinical change observed after IN inoculation. In summary, IN inoculation with BoHV-4 isolate SD16-38
in this study was followed by transient replication, viremia, and virus shedding in nasal secretions but did not result in overt respiratory disease.

Previous data have shown that environmental conditions may also influence BoHV-4 pathogenesis [20, 24]. While a few studies used the same methodology and viral strain to inoculate calves of roughly the same age, diverging results were observed regarding the sites of BoHV-4 persistence/latency. The first study, with nine animals, found no evidence of the presence of BoHV-4 DNA in neurological tissues of any of the animals after day 28 pi – a period of time that could be considered sufficient to allow the establishment of viral persistence/latency in neurological tissues [20]. On the other hand, in a second study, in two animals maintained for 62 days, BoHV-4 DNA was detected in neurological tissues by nPCR [24]. In the present study, both animals euthanized on day 35 pi had viral DNA in the trigeminal ganglia and the retropharyngeal lymph node, as determined by nPCR and RNAscope ISH. These results suggest that intranasal inoculation with BoHV-4 led to effective infection and establishment of persistence/latency in neuronal and lymphoid tissues, corroborating previous observations [26]. Interestingly, we observed an increasing number of tissues positive for viral DNA over the course of the study. BoHV-4 DNA was initially detected at the inoculation site and draining lymph nodes. Later, at day 35, most of the tissues were positive for BoHV-4 DNA, although no infectious virus was recovered from nPCR-positive tissues. These results suggest slow and progressive infection of tissues or perhaps infiltration of tissues with persistently/latently infected monocytes/macrophages. Detection of virus and/or viral DNA in the buffy coats supports dissemination of the virus associated with leukocytes. The transient detection of BoHV-4 DNA in lymphoid tissues of most inoculated animals on days 5 and 10 pi suggests that these tissues might be targets for acute BoHV-4 replication, whereas viral DNA detection of both lymphoid and neural sites (TGs) at day 35 pi suggests both tissues as sites of BoHV-4 persistence/latency. It is conceivable that, following IN inoculation and replication, transient viremic spread of the virus occurs, resulting in subsequent infection of neural and lymphoid tissues and establishment of persistence/latency. Long-term viral persistence in lymphoid tissues has been demonstrated in initial studies of BoHV-4 pathogenesis in cattle [25, 35]. Likewise, sensory nerve ganglia (e.g., trigeminal ganglia) of naturally infected cattle have been shown to harbor BoHV-4 DNA [37, 38]. Thus, current evidence suggests that lymphoid and neural tissues may serve as sites of BoHV-4 persistence/latency [25, 35].

The route of BoHV-4 inoculation and the level of cell-associated viremia seem to play a role in the clinicopathological outcome of infection. In a previous study, 7-day-old calves inoculated with the European isolate Movar 33/63 via the IN and intratracheal routes developed bronchitis and nasal discharge [20]. Virus shedding was detected in nasal and ocular discharges for 2 weeks postinfection, and viremia was detected up to 12 days pi. The development of clinical signs and long-term shedding, in contrast to the present study, may reflect differences in virulence among strains, inoculation methods, and/or host factors. Intratracheal inoculation likely facilitates infection of lung epithelial cells, monocytes, and macrophages and may bypass part of the intrinsic and innate immune responses, leading to clinical signs and a higher level of cell-associated viremia. Bypassing the intrinsic and innate immune defenses has been achieved by intratesticular BoHV-4 inoculation of bulls, which led to conjunctivitis between days 14 and 18 pi and azoospermia between days 31 and 51 in one of the five animals inoculated [22]. Shedding through ocular secretions was also detected after day 19 pi.

The humoral response to BoHV-4 infection in cattle is characterized by low levels of antibodies. Some studies have shown that the neutralizing activity of antibodies against BoHV-4 appears to be strain-specific, with some viral strains being readily neutralized, while others are capable of escaping virus neutralization [39]. These differences could be a result of different glycosylation profiles of the major viral glycoproteins involved in cell binding and entry [39], or possibly, of altered tropism of the virus for epithelial cells or for myeloid CD14+ cells (monocytes, macrophages, and some granulocytes), a function that is regulated by alternative splicing of the entry glycoprotein gp180, which leads to shielding of vulnerable entry epitopes [40]. The virus neutralization results obtained here, demonstrating neutralizing antibody titers of 4 and 16 in the two animals that were maintained until day 35 pi, are consistent with the low neutralizing activity of antibodies to BoHV-4. This is an interesting property of the virus, which may allow its broad circulation in the cattle population and could be exploited to develop BoHV-4 as a viral vector platform for vaccine delivery in cattle [41].

In summary, the study described here provides evidence of subclinical BoHV-4 infection in calves and the ability of the virus to establish latency in neuronal and lymphoid cells following intranasal inoculation. The increased frequency of isolation of BoHV-4 in cattle herds in the Midwest region of the USA seems not to be due to dissemination of strains with increased virulence, and further investigations are required to fully understand the role of BoHV-4 in disease. It is conceivable that BoHV-4, similar to other agents involved in the bovine respiratory disease complex, may act as a cofactor in disease production. Additionally, given the lack of overt disease in our study and other studies, the possibility that BoHV-4 may not be a primary disease-causing agent in cattle also has to be considered. Frequent isolation of the virus
from healthy animals or from those with various inflammatory conditions may well be a result of the virus tropism, replication, and persistence/latency in monocytes, which function during inflammation to clear damaged cells.

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Author contributions Conceived the study: SFM, EFF, DGD. Tested experimental samples: FVB, SMF, MM, RPD, SS, MVP, AB, AM. Data analysis and result interpretation: FVB, SMF, MM, JDN, JFR, MVP, EFF, DGD. Manuscript writing: FVB, SMF, MM, EFF, DGD. All authors have reviewed and edited the manuscript.

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Data availability The complete genome sequences generated in this study can be found in the GenBank database under accession numbers MN551083 and MN551084.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval All animals were handled in accordance with the Animal Welfare Act Amendments (7 U.S. Code §2131 to §2156), and all study procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the National Animal Disease Center (ARS-2016-572) or by the Ethical Committee at UFSM (CEUA/UFSM protocol number 034/2014).

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