The First Full-Genome Characterization and Phylogenetic Analysis of a Potential Recombinant Bovine Herpesvirus Type 1 Isolated in China

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Abstract

Bovine herpesvirus type 1 (BHV-1) is a virus of causing bovine respiratory disease that poses significant threat to the cattle industry. The prevalence of BHV-1 has recently increased in China. However, information about the prevalent isolates is scarce. In this study, we identified a novel strain of BHV-1, referred to as BHV SHJS, isolated from nasal swabs of Holstein cows in 2020 of China. The genome of BHV SHJS is 1,35,102 bp in length and highly similar to SP1777 (KM258883.1) strain with an identity of 99.64 % . Mutations, insertions, or deletions mainly occur in UL12, UL19, UL27, UL37, UL42, UL44, UL46, UL47, US6, US7, and US8 relative to the different genomic coordinates. Phylogenetic analyses of immunogenic gene (gB, gC, and gD) revealed that BHV SHJS and other China outbreak strains were displayed on different branches with commonly used vaccine strains. Recombination analysis shown that BHV SHJS could persist even with BoviShield IBR MLV and Arsenal IBR MLV vaccines, suggesting that these vaccines should not completely prevent BHV SHJS infection. These analyses partially explain why the vaccines only partially protect against BHV-1 outbreaks in China. In conclusion, we identified a novel and potential recombinant BHV-1 strain and for the first time characterized BHV-1 genome with a different evolutionary origin from that of known strains prevalent in China. This study will enrich our knowledge regarding BHV outbreak strains in China and contribute to the prevention and pathogenic studies of BHV-1.

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Abstract

Bovine herpesvirus type 1 (BHV-1) is a virus of causing bovine respiratory disease that poses significant threat to the cattle industry. The prevalence of BHV-1 has recently increased in China. However, information about the prevalent isolates is scarce. In this study, we identified a novel strain of BHV-1, referred to as BHV SHJS, isolated from nasal swabs of Holstein cows in 2020 of China. The genome of BHV SHJS is 1,35,102 bp in length and highly similar to SP1777 (KM258883.1) strain with an identity of 99.64 %. Mutations, insertions, or deletions mainly occur in UL12, UL19, UL27, UL37, UL42, UL44, UL46, UL47, US6, US7, and US8 relative to the different genomic coordinates. Phylogenetic analyses of immunogenic gene (gB, gC, and gD) revealed that BHV SHJS and other China outbreak strains were displayed on different branches with commonly used vaccine strains. Recombination analysis shown that BHV SHJS could persist even with BoviShield IBR MLV and Arsenal IBR MLV vaccines, suggesting that these vaccines should not completely prevent BHV SHJS infection. These analyses partially explain why the vaccines only partially protect against BHV-1 outbreaks in China. In conclusion, we identified a novel and potential recombinant BHV-1 strain and for the first time characterized BHV-1 genome with a different evolutionary origin from that of known strains prevalent in China. This study will enrich our knowledge regarding BHV outbreak strains in China and contribute to the prevention and pathogenic studies of BHV-1.

Key words: Bovine herpesvirus type 1, full-genome, potential recombinant virus; China

1 I ntroduction

Bovine herpesvirus type 1 (BHV-1) was a key pathogen causing an contagious bovine respiratory disease or abortion, that pose a threat to the cattle industry and significant economic losses (Brake & Studdert, 1985; Hodgson et al., 2005; Jones & Chowdhury, 2007). In China, the prevalence of BHV-1 presented fluctuations of 5 % to 68.7% from 2012 to 2017 in cattle (Mo, 2017; Pi, 2014; Zhong, 2012). In 2018, a meta-analysis showed prevalence of up to 40% in China (Chen et al., 2018). Interestingly, a phenomenon has been observed in the Chinese cattle farms where the rate of abortion in cows has been rising after vaccination.

BHV-1 belongs to the family *Herpesviridae* , subfamily *Alpha herpesvirinae* (Nandi, Kumar, Manohar, & Chauhan, 2009). BHV-1 exhibits a double-stranded DNA of approximately 135 kb in size (Engels, Loepfe, Wild, Schraner, & Wyler, 1987). The BHV-1 genome contains a unique long sequence region (UL), a unique short sequences region (US), an internal repeat sequences region (IR) between UL and US, and an inverted terminal repeat sequences region (TR) (Leung-Tack, 1994, d'Offay, 2016). It was composed of about 73 open reading frames (Engels, 1986). Glycoprotein B (gB), glycoprotein C (gC), and glycoprotein D (gD) encoded by UL27, UL44, and US6, respectively, were the major attachment proteins, which could induce a high level of neutralizing antibodies against BHV-1 (Babiuk et al., 1987; Liang, Babiuk, van Drunen Littel-van den Hurk, Fitzpatrick, & Zamb, 1991; van Drunen Littel-van den Hurk, Gifford, & Babiuk, 1990).

Modified live virus (MLV) vaccines and inactivated or attenuated vaccines are used against BHV-1 conventionally (Deshpande et al., 2002; Marawan et al., 2021; O'Toole, Miller, Cavender, & Cornish, 2012). However, they only partially protect from wild-type BHV-1 infection (Fulton, d'Offay, Dubovi, & Eberle, 2016; Fulton et al., 2015; O'Toole, Chase, Miller, & Campen, 2014). We were perplexed when we discovered that cow abortion rates increased following vaccination in China.

It has been reported that homologous recombination often occurs in the herpesvirus species: Pseudorabies virus (PRV), Varicella-zoster virus, and herpes simplex virus type 1 (HSV-1) (Bo et al., 2021; Kintner, Allan, & Brandt, 1995; Norberg et al., 2015; Schynts, Meurens, Detry, Vanderplasschen, & Thiry, 2003; Thiry et al., 2005). Thus, there was a huge responsibility placed on virus infection prevention. Recombination events between BHV-1 and BHV-5 are also reported (Meurens et al., 2004). Recombination of live-attenuated BHV-1 was first documented in Australia in 2008 (Lee et al., 2012). There are no reports of recombination events in cattle infected with BHV-1 in China.

In 2020, a local farm of Holstein cows developed severe respiratory inflammation and abortion symptoms in

Shanghai, China. We suspected that those cows might be infected with BHV-1. In this study, we isolated a novel strain of BHV-1 from the nasal swabs. Then, the biological characteristics of the virus were systemically investigated; The genome of the isolate was sequenced, organized, and analyzed. According to recombination analysis, the isolated virus was a novel recombinant virus—to our knowledge, this is the first instance in China. This study will contribute to investigating the evolution of BHV-1 and aid in its future prevention or management.

2 Materials and methods

2.1 Cells and virus isolation

Madin-Darby bovine kidney cells (MDBK) were purchased from the American Type Culture Collection and Stored in the laboratory. Cells were incubated using Dulbecco's Modified Eagle's Medium (DMEM) (BI, China) with 8% fetal bovine serum (FBS) (Pan Biotech, Germany) at 37 °C in 5% CO_2incubator. Thirty-three nasal swabs were collected from Holstein cows from a bovine farm in Jinshan District, Shanghai, China, in 2020. BHV-1 US7 positive samples were homogenized with DMEM and centrifuged using 6,000 × g for 5 min. The supernatant was filtered by filter unit and then incubated with MDBK cell monolayers grown for virus isolation by monitoring cytopathic effects (CPEs).

2.2Polymerase chain reaction (PCR)

DNA was extracted from nasal swabs and the supernatant of the infected cells using the Ezup column viral DNA kit (Sangon Biotech, China). Specific primers were designed to amplify BHV-1 US7, US8, UL48, and UL49 genes (Table S1). PCR reactions were amplified using DNA polymerase (Vazyme Biotech, China) under the conditions: 95 °C for 3 min, 35 cycles at 95 °C for 15 s, 68 °C for 1 min, and 72 °C for 2 min (Waters & Shapter, 2014).

2.3Plaque formation assay

MDBK cells were infected with the virus of ten-fold serial dilutions. Following 2-hour incubation, the cells were washed thrice using PBS. Then, infected cell was overlaid using 1% agarose (Invitrogen, USA) that was low-melting-point and supplemented with 2% FBS. Then, cells overlaid with agarose were placed 4 °C for 5 min. Next, cells were Following incubation at 37 °C, cells were observed whether the plaques were formed. Fifty plaques were randomly selected for virus (Moe, Lambert, & Lupton, 1981), The diameter was measured using software of Image J (National Institutes of Health).

2.4 Growth curve and virus titer assays

The growth kinetics of the virus was determined by infecting MDBK cell monolayers with the virus at a multiplicity of infection (MOI = 1) for 2 h. Then, cells were washed thrice with PBS and replaced with DMEM with 2% FBS. The cells and supernatant of infection were collected at different time point (6, 12, 24, 36, and 48 h). Following three freeze-thaw cycles at -80 °C, the cells and supernatant were prepared at centrifugation of 12,000 × g for 3 min. The $TCID_{50}$ of these samples were determined using 96-well plates coated with MDBK cell monolayers. Cells were infected with supernatant by10-fold dilutions from 10^{-1} to 10^{-9} for sample. The CPEs of the cells were observed daily. The $TCID_{50}$were determined according to the Reed and Muench method (REED & MUENCH, 1938).

2.5Genomic sequencing and

phylogenetic analysis

Viral DNA was extracted using Viral DNA Kit (Omega, USA) from supernatant of infected cells referring to the instructions. The full genome sequence was obtained using the second-generation sequencing platform of the Gen-Script company. The full genome sequence was compared with the published BHV-1 complete genomes in the NCBI database. The full genome of the isolated BHV-1 strain was analyzed using the viral genome with the highest homology as reference. Phylogenetic analyses based on BHV-1 UL44 (gC), UL27 (gB), and US6 (gD) sequences were performed using MEGA software (version 7.0.26). The reference

sequences of UL44 (gC), UL27 (gB), and US6 (gD) genes retrieved from GenBank for phylogenetic analysis are listed in Table 1.

2.6Recombination analysis, alignment, and prediction of structure

RDP (version 3.5.1) software was used to identify recombination analysis using RDP and Boot-scan method based on published vaccine strain genome in the NCBI database. The amino acid sequences were aligned by website of alignment (https://www.ebi.ac.uk/Tools/msa/clustalo/). Protein-encoded UL42 and UL46 secondary structures were predicted by PredictProtein (https://predict-protein.org/). Protein domains were predicted by SMART (https:// smart. Embl.de/).

2.7 Western blotting analysis

The cells extracts were processed using SDS buffer and subjected to PAGE (YA Mei, China). At room temperature, proteins were electrophoresed to polyvinylidene fluoride (PVDF) membranes (Pall Corp, China) and blocked with 5% non-fat milk in PBS for 1 h. Membranes were incubated overnight at 4 °C with BHV-VP22 polysera (prepared in our lab) and β-actin antibody (CST, USA). The next day, membranes were washed thrice in PBST and incubated with secondary antibody (Sigma, USA). Membrane signals were developed using the ECL kit (Singke, China) and detected using a Pierce SuperSignal West Pico Chemiluminescent Substrate using a Chemiluminescence Immunoassay image analysis system (Tanon5200, China).

2.8 Immunofluorescence assay (IFA)

Viral replication was investigated by growing the MDBK cell monolayer on coverslips at 6, 12, 18, 24, 36, and 48 hours post-infection (hpi). Monolayer was incubated when that fixed with 4% formaldehyde at 37 °C for 35 min. Then, cells were washed with PBS buffer and blocked with PBS with 5% milk of non-fat at 37 °C for 30 min. Cell were washed with PBS buffer and incubated with BHV-VP22 polysera (1:500 dilution) for 45 min, followed by washing with PBS and staining with DAPI (4',6-diamidino-2-phenylindole). Images were captured under an ECHO fluorescence microscope (RVL-100-g, USA).

2.9Real-time quantitative PCR (qPCR)

The profiles of TNF-α, IL-1β, and IL-6 mRNA was detected using qPCR in response to virus infection. MDBK cells were infected with isolated strains (MOI = 1) for 6, 12, 18, 24, 36, and 48 hpi. At indicated time points post-infection, the MDBK cells were harvested at specified point time for RNA isolation. Total RNA was extracted using RNAprep Pure Cell Kit (TIANGEN, China) and reverse transcribed to cDNA using Reverse Transcriptase (Takara. China). The transcriptional levels of cytokines were determined by RT-qPCR using qPCR Mix SYBR Green I (Singke, China). The amplification programs were 95 °C for 1 min, followed by 40 cycles: 95 °C for 10 s and 60 °C for 32 s. Fold changes were calculated by the ΔΔCT method in mRNA expression level. The primers designed was presented in Table S2.

2.10 Statistical analysis

Data are collated and presented as mean ± standard deviation (SD). Differences of the groups were determined by t-test with Graph Pad Prism software (version 7.0). Ap -value smaller than 0.05 was considered statistically significant ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

3 Results

3.1Isolation and biological characteristics of isolated virus

Four samples were identified as positive-BHV from 33 nasal swabs (Figure S1). Positive samples were inoculated on MDBK cells to obtain the virus. Only one sample showed visible CPEs with round and enlarged cells shrunk or detached at 48 hpi (Figure 1a). After three purification cycles, 1 to 2 mm diameter plaques were formed in infected cells compared with the mock group (Figure 1b). Three fragments were amplified from the infected cells by PCR (Figure 1c). Further sequence analysis revealed that the three amplified

fragments showed high similarity to BHV-1 US7/US8 (3156bp), UL48 (1524bp), and UL49 (776bp) (Figure S2), respectively. The data suggested that the isolated virus was BHV-1, referred to as BHV SHJS.

To investigate the biological characteristics of isolated strain, the growth curve was determined on cells infected with the purified BHV SHJS (MOI = 1) at 6, 12, 24, 36, and 48 hpi. The results showed that the virus titer was 8.02×10^7 $TCID_{50}$/mL at 48 hpi (Figure 1d). Western blotting and IFA analysis showed that virus infection could be detected at 12 hpi and 6 hpi, respectively (Figure 1e, f). To explore whether BHV SHJS infection affects the transcription levels of TNF-α, IL-1β, and IL-6. The mRNA levels of IL-1β, IL-6, and TNF-α was determined by quantifying in the infected MDBK cells. The results showed that these mRNA levels significantly increased, reaching the highest levels at 36, 18, and 36 hpi, respectively, after significantly decreasing (Figure 1g).

3.2 Characterization of BHV SHJS genome

The full genome of BHV SHJS was sequenced by second-generation sequencing. After assembly, a 1,35,102 bp genome of BHV SHJS was obtained. The average coverage depth of the assembled sequences was 3332.6 ×. Genome of BHV SHJS has been submitted to NCBI (GenBank accession number: OP035381).

Although BHV-1 is prevalent in China, no full genome map of the BHV-1 isolates has been reported. We found that the BHV SHJS genome was collinear with BHV-1 K22 (KM258880.1) genome (d'Offay, Eberle, Fulton, & Kirkland, 2016). The full genome of the BHV SHJS virus included a unique long sequence region (UL), a unique short sequences region (US), an inverted repeat sequences region (IR), and an inverted terminal repeat sequences region (TR). Sequence analysis showed that the BHV SHJS genome contains 76 ORFs, of which 32 ORFs encode viral structure proteins. Five ORFs could encode products of related enzymes; Six ORFs could encode products of regulatory function-related proteins. These are marked in different colors in map of BHV SHJS genome (Figure 2).

3.3 Analysis of BHV SHSJ genome

We found that the BHV SHSJ genome shares 98.57% to 99.64% nucleotide identity with twenty-five BHV-1 strains (including American, Australian, and Indian strains) available in GenBank, and the highest nucleotide identity (99.64%) was seen with SP1777 (KM258883.1). Genome sequence alignment showed that mutations, insertions, or deletions mainly were observed in BHV SHJS UL12, UL19, UL27, UL37, UL42, UL44, UL46, UL47, US6, US7, and US8 genes compared to its different genomic coordinates (Figure 3a). Amino acid alignment of these sequences showed that 41 Val (V) and 204 Gly (G) were mutated to Ile (I) and Ser (S) in BHV SHJS US8 (gE), respectively (Figure S3A). Two insertions, including 333 Ser (S) and 334 Ala (A), and one mutation at 369 H (His) that mutated to Q (Gln) were observed in BHV SHJS UL42 (Figure S3B). Two deletion sites at 456 to 457, 552 to 571, and 648 to 653, two insertion regions located at 611 to 614 and 649 to 656, and one mutation site at 514 F (Phe) that mutated to C (Cys) were found in the sequence of BHV SHJS UL46 (Figure S3C). The data suggested that the genomic differences of BHV SHJS compared with that of the previously reported BHV strains occur at US8, UL42, and UL46.

3.4 Phylogenetic analysis of BHV SHSJ genome and gE

The phylogenetic tree was constructed and analyzed to explore the genetic and evolutionary status of BHV SHJS. Phylogenetic analysis of the BHV SHJS genome showed that BHV SHJS was clustered with the K22 strain and far away from the other BHV-1 strains (Figure 3b). Due to the lack of a full genome for BHV-1 strains in China, we used the published BHV-1 gE gene based on the nucleotide sequences to construct the phylogenetic tree of gE. Analysis of gE showed that BHV SHJS was displayed on a separate clade far away from the previously reported MN06 and NM14 strains isolated in China and the strains reported in the USA, India, and Australia. These results suggested that China's newly identified BHV SHSJ might be a new outbreak strain (Figure 3c).

3. 5 Phylogenetic analysis based on gC

BHV UL44(gC) has been previously used for phylogenetic analysis (Chowdhury, 1995, 1997; Zhou et al.,

2020). Due to high variability in the N-terminal region, gC has also been used to determine the genotypes of BHV-1 and BHV-5 (Spilki et al., 2005; Traesel, Bernardes, Spilki, Weiblen, & Flores, 2015). The phylogenetic tree of gC constructed based on 48 reference nucleotide sequences showed that BHV gC was clustered in three major clades, including genotypes I, II, and V (Figure 4). Clade I was further divided into two separate lineages: lineage 1 was mainly composed of BHV SHJS (2020), Liaoning strains (2018, 2019), Jilin strains (2016, 2017, 2019), Heilongjiang strains (2018, 2019), and Inner Mongolia strains (2019) isolated in China and strains isolated in Australia (2001); lineage 2 consisted of strains isolated in America, India (1976), Switzerland (1992). Clade II consisted of strains isolated in Germany. Clade V contained strains isolated in Brazil (1999, 2007) and Uruguay (2012). BHV SHJS was clustered with SM023 (1986) on one branch in the lineage close to strains isolated in China. Based on these analyses, we confirmed that BHV SHJS belongs to genotype I.

3.6Phylogenetic analysis of major immunogenic genes

BHV-1 UL27(gB), UL44 (gC) , and US6 (gD) encode the key immunogenic proteins that could induce high levels of neutralizing antibodies, with the highest antibody levels seen with gB (Babiuk et al., 1987; Liang et al., 1991; van Drunen Littel-van den Hurk et al., 1990; Yoon & Eo, 2007).

To investigate whether the available BHV-1 vaccines could protect against BHV SHJS, outbreaks was studying by conducting a phylogenetic analysis based on these genes of the available BHV strains and commercial vaccine strains. Phylogenetic analysis of gB and gD showed that the two were clustered in three clades (1, 2, and 3) (Figure 5a, b). For the phylogenetic tree based on gB, BHV SHJS was closely clustered with China IBRV (2009, 2018), indicating that IBRV (2009, 2018) might be the closest ancestor of the BHV SHJS. Clade 1 consisted of BHV SHJS and other strains isolated in China, the USA, and Egypt, far from the available vaccine strains displayed in clade 2 (Figure 5a). For the phylogenetic tree based on gC, the strains obtained in China, including BHV SHJS and the available vaccine strains, were displayed on two different lineages, although they all belonged to genotype I (Figure 4). In the case of the phylogenetic tree based on gD, BHV SHJS and the vaccine strains were displayed on two different clades, while some other strains isolated in China were presented on the same clade with the vaccine strains (Figure 5b). These analysis suggested that the available vaccines could not provide protection against BHV SHJS and may only provide partial protection against other BHV-1 strains in China. This can also partially explain why BHV-1 vaccine strains only provide partial protection from the new outbreaks of genotype I in China or the USA.

3.7Recombination analysis of BHV SHJS

MLV vaccines and inactivated and attenuated vaccines were also widely used against BHV-1 infections (Deshpande et al., 2002; Marawan et al., 2021). Interestingly, the rate of abortion is increasing in cows after vaccination in bovine farms in China. we speculated that it might be caused by homologous recombination.

To verify this hypothesis further, recombination analysis of BHV SHJS with nine commercial vaccine strains was performed. The analysis showed that BHV SHJS has a potential recombinant region in the genome from 11,882 to 38,620 positions (Figure 6a) and parents were found that were BoviShield IBR MLV and Arsenal IBR MLV vaccine strain on the evolutionary relationship of recombinant events (Figure S4A and B). Event reliability was further verified by analyzing the recombination score and Boot-scan. The recombination score was 60.3% (Figure S4C). The Boot-scan analysis showed that the recombinant region also occurred at the similar position in the BHV SHJS genome (Figure S4D). This analysis suggested that the recombination analysis was credible.

Alignment of the recombinant region showed that the virus included twelve ORFs (UL37 to UL48), and UL46, UL42, UL41, and UL37 are potential exchange points (Figure 6b). Alignment of complete nucleotide sequences of exchange points showed that UL46, UL42, and UL37 would be subjected to insertion or mutation, and UL41 would undergo mutation in vaccine strain (Figure 6c). The opposite, BHV SHJS might also be derived from the recombination of vaccine strains with wild strains (Figure 6b). These analyses demonstrated that BHV SHJS could be derived from recombination, and UL46, UL42, UL41, and UL37 nucleotides are potential exchange points if commercial BoviShield IBR MLV and Arsenal IBR MLV vaccines were used

against BHV-1 infection. These also partially explain the phenomenon of the increasing rate of abortion in cows after vaccination in bovine farms in China.

4 Discussion

Recently, infection and transmission of BHV-1 have been common in cattle in China (Mo, 2017; Zhou et al., 2020). In 2018, the prevalence was 40% in cattle occurred in China (Chen et al., 2018). However, information about the prevalent of Chinese isolates is scarce. In 2020, we isolated a BHV strain, referred to as BHV SHJS, from the nasal swabs of Holstein cows in Shanghai, China. Complete genome analyses showed that BHV SHJS has the highest nucleotide identity (99.64%) with SP1777 (KM258883.1) and contains UL, US, IR, and TR regions (Figure 2). Phylogenetic analysis demonstrated that BHV SHJS belongs to genotype I and presented as a new outbreak field strain in China.

TNF-α, IL-1β, IL-6 play an vital role in inflammatory and immune responses to antiviral infection (Beutler & Cerami, 1986; Bradley, 2008; Horiuchi, Mitoma, Harashima, Tsukamoto, & Shimoda, 2010; Hsieh et al., 1993; Komastu, Ireland, & Reiss, 1998; Pasquereau, Kumar, & Herbein, 2017). In response to some herpesvirus infections, expression changes of inflammatory cytokines were observed previously. For instance, in tracheal epithelium cells, BHV-1 Cooper infection could increase the transcription and expression level of TNF-α (Burucúa et al., 2019). In peripheral blood mononuclear cells or tracheobronchial lymph nodes, the level of IL-1β mRNA increased during BHV-1 infection (Leite, Kuckleburg, Atapattu, Schultz, & Czuprynski, 2004). To date, the transcription and expression of IL-6 during BHV-1 infection are not reported. Interestingly, in the participants' serum after HSV-1 or CMV infection, higher concentrations of IL-6 were detected by ELISA (Noronha et al., 2021). In HSV-infected mice, after 5 days, the expression of IL-6 also increased (Dhanushkodi et al., 2020). We found that transcription levels of IL-6 were increased for 24 hpi and 36 hpi in BHV SHJS infected cells. Trends of increasing levels of TNF-α and IL-1β are consistent with the previous reports. This provided a reference for revealing mechanisms of inflammation due to infectious diseases caused in the future.

We also found that a mutation, deletion, or insertion occurs in some amino acids of BHV SHJS UL42 and UL46 proteins (Figures S3B, S3C). BHV-1 UL46 has been reported to impact viral replication (Hou, Zhao, He, He, & Wang, 2019). UL46 of herpesvirus HSV has an important role in antiviral innate immune response (You et al., 2019). There are few in-depth studies on BHV-1 UL46 and UL42 proteins. We focused on speculating the function of UL46 and UL42 encoded proteins and the influence of site changes. We predicted the structure of proteins and showed that UL42 encoded protein consisted of 17.65% alpha helix (H), 26.72% extended β strand (E), and 55.64% loop (L) (Figures S5A), and UL46 encoded protein consisted of 44.74% alpha helix (H), 1.8% extended β strand (E), and 53.4% loop (L) (Figure S5A) on secondary structure. UL42 encoding protein contained Malic-M, PKS-KR domains (Figure S5B), and UL46 encoding contains HhH1, PKS-TE, ICA69, and GDNF domains (Figure S5C). Insertions of BHV SHJS UL42 occurred in the predicted PKS-KR domain, which was associated with bacterial polyketide synthases and catalyzes. Deletions and mutations of BHV SHJS UL46 occurred in an Islet cell autoantigen ICA69 domain and GDNF domain which is a potent survival factor for sympathetic, sensory, and central nervous system neurons (each functional domain description is derived from the database on prediction). Based on these predictions, we speculated that the functions of these two proteins might be similar to these functional domains. However, mutation, deletion, or insertion of these domains might influence protein functions.

Currently, commercial MLV vaccines, inactivated or attenuated live vaccines, and recombinant subunit vaccines are used to immunize cattle to gain protection against BHV-1 (Biswas, Bandyopadhyay, Dimri, & Patra, 2013; Cowley, Clegg, Doherty, & More, 2011; Fulton, d'Offay, & Eberle, 2013; Gonçalves et al., 2021). However, these vaccines only partially protect against BHV-1, as observed in China or the USA (O'Toole et al., 2012). Phylogenetic analyses of immunogenic genes (gB, gC and gD) revealed that BHV SHJS and some other Chinese outbreaks, and some American vaccine strains were displayed on different branches. Based on these results, we speculate that the available vaccines in China may not protect BHV SHJS and may only provide partial protection against other BHV-1 strains in China. This can also partially explain why BHV-1 vaccine strains only provide partial protection from the new outbreaks of genotype I in

China or the USA.

Recombination analysis demonstrated that BHV SHJS could be derived from recombination if commercial BoviShield IBR MLV and Arsenal IBR MLV vaccines were used against BHV-1 infection. Another opposing observation suggested that BHV SHJS might be derived from the recombination of vaccine strains with wild strains (Figure 6b). These partly explained the phenomenon of the increasing rate of abortion in cows after vaccination in bovine farms. In China, the epidemic of BHV-1 is difficult to control and may be caused by the virulence of vaccines that becomes stronger when recombination occurs between vaccine strains and wild type viruses. In the future prevention, purchased BoviShield IBR MLV and Arsenal IBR MLV vaccines with should be prevented to escape from recombination. We recommend choosing different vaccines or vaccines developed based on the BHV SHJS strain to avoid BHV SHJS outbreaks in the future.

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COMPETING INTERESTS

There have no competing interests for authors.

ETHICS STATEMENT

There was no animal experiment was performed in this experiment.

AUTHOR CONTRIBUTIONS

WG, HC, SJ, and YQ conceived and designed the experiments. WG and JX performed the experiments. LJ and WG analyzed the data. HC and YQ helped with reagents/ materials/ analytical tool. WG, HC and LJ wrote the manuscript

DATA AVAILABILITY STATEMENT

The data are available from the corresponding author on reasonable request.

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Figure Legends

FIGURE 1 Isolation, identification, and biological characterization of the isolated BHV SHJS strain.

(a) Isolation of virus on MDBK cells. Virus isolation from the nasal swab. CPE was observed on BHV SHJS-infected and mock cells. (b) Plaque assay. MDBK cells were infected with the isolated virus. (c) Amplification of the conserved gene in infected cells. The fragments were amplified from extracted DNA from the supernatant of infected cells using specific primers for BHV-1 US7/US8 (lane 1), UL48 (lane 2), UL49 (lane 3). (d) Growth curves of BHV SHJS in MDBK cells. Cells were infected with BHV SHJS (MOI = 1). The $TCID_{50}$ values were determined by the Reed and Muench method. Dynamic changes of viral infection were verified by western blotting (e) and IFA (f). The cells infected with the virus (MOI = 1) were incubated and recognized using VP22-specific antibodies. (g) The mRNAs level of cytokine in infected cells. Cells were infected with BHV SHJS (MOI = 1) for 6, 12, 24, 36, and 48 hpi. The mRNA levels of TNF-α, IL-6, IL-1β were quantified by qPCR. A p-value smaller than 0.05 was considered statistically significant ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

FIGURE 2 Organization of BHV SHJS full genome map.

Genome of BHV SHJS contains unique long sequence region (UL) and unique short (US) sequences region. An internal repeat sequence region exists between the UL and US region, called the IR region. Inverted repeat sequences, called the TR region, are present at the end of the genome US region.

FIGURE 3 Phylogenetic analysis of the complete genome and US8 (gE) sequence.

(a) Analysis of the complete genome sequence for BHV SHJS. (b) Phylogenetic analysis of BHV SHJS full genome sequence determined using the Neighbor Joining method in Molecular Evolutionary Genetics on NCBI.

(c) Phylogenetic analysis of US8 (gE) sequence using Maximum like-hood method by MEGA software.

FIGURE 4 Maximum-likelihood analysis of BHVSHJS UL44 gene-based sequence. Forty-eight sequences of the BHV-1 strains with complete UL44 sequences are available in GenBank and were compared using MEGA7 software. The sequences were aligned using the Clustal W method.

FIGURE 5 Maximum-likelihood analysis of BHVSHJS UL27 (gB) and US6 (gD) gene-based sequence. Fifty-six nucleotide sequences of BHV strains UL27 and 69 sequences of US6 are available in GenBank and were compared using MEGA7 software. The sequences of UL27 (A) and US6 (B) were aligned using the Clustal W method.

FIGURE 6 Recombinant analysis of BHV SHJS with vaccine strains. (a) BHV SHJS recombinant region was located in the genome between 11,882 and 38,620 bp. (b) BHV SHJS ORFs in the recombination regions determined by alignment using the Clustal Omega website. Recombination ORFs were present in UL46, UL42, UL41, and UL37, and are marked in pink. Non-recombination ORFs are marked in orange. (c) Specific exchange positions and ORFs contained in the recombination regions. ORFs of exchange points are marked in pink in the potential recombinant region. In China, there is no whole-genome sequence of BHV-1 published on GenBank. BHV-1 K22 was selected as a wide strain reference due to close evolutionary relationships.

Supporting information

The First Full-Genome Characterization and Phylogenetic Analysis of a Potential Recombinant Bovine Herpesvirus Type 1 Isolated in China

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TABLE S1 PCR primer sequences

Name	Sequence
BHV-VP16-F	5'-CCGGAATTCATGAGCGGGCGCATAAAA-3'
BHV-VP16-R	5'-CGCTCGAGGAAGTCCAGCAGCTGGTT-3'
BHV-VP22-F	5'-GGAATTCATGGCCCGGTTCCACAGG-3'
BHV-VP22-R	5'-CCCAAGCTTGCCGCGAAGGCGGCTTTC-3'
BHV-US7-F	5'-CCGGAATTCATGCGGTGCCTGTTGCTCTGG-3'
BHV-US7-R	5'-GCTCTAGATTATTCTTCGCTGATGGTGGCGAGGGG-3'
BHV-US8-F	5'-CCGGAATTCATGCAACCCACCGCGCCGCCC-3'
BHV-US8-R	5'-GCTCTAGACTAGCGGAGGATGGACTTGAGTCGCGC-3'

TABLE S2 qPCR primer sequences

Name	Sequence
bovine-qPCR-actin-F	5'-AAGTACCCCATTGAGCACG-3'
bovine-qPCR-actin-R	5'-GAAGGTCTCGAACATGATCTGG-3'
bovine-qPCR-IL-1β-F	5'-AGTGCAAACTCCAGGACAGA-3'
bovine-qPCR-IL-1β-R	5'-GATACCCAAGGCCACAGGAA-3'
bovine-qPCR-TNF-α-F	5'-GGCTTTACCTCATCTACTCACAG-3'
bovine-qPCR-TNF-α-R	5'-AGACTGGATGTTGACCTTGG-3'
bovine-qPCR-IL-6-F	5'-GATGACTTCTGCTTTCCCTACC-3'
bovine-qPCR-IL-6-R	5'-TTTCTGCCAGTGTCTCCTTG-3'

FIGURE S1. Results of PCR amplification and alignment products with BHV conserved genes. Fragments were obtained successfully by PCR amplification from DNA extraction of the Thirty-three Nasal swabs samples (NO.1 to 33 in lane) using specific primers for BHV-1 US7. Fragment of positive control was amplified from DNA of BHV-1 batha, which was marked with positive label in lane. Alignment result of PCR fragments sequence with reference strain (KM258882.1) BHV-1 US7.

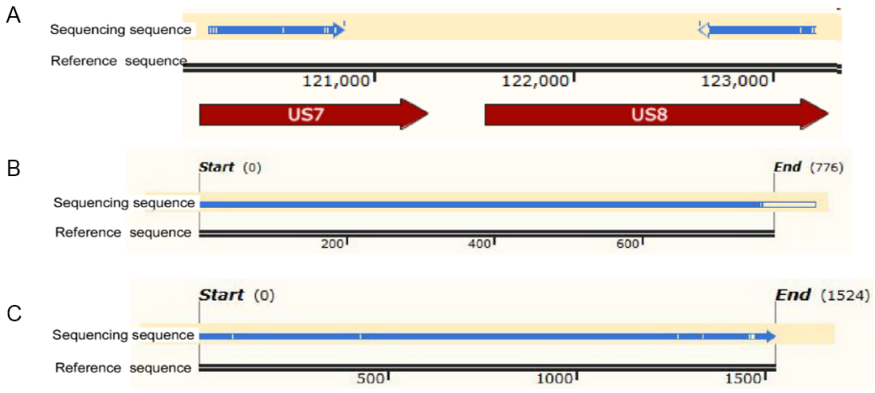


FIGURE S2. Alignment results of sequence of PCR amplified products with BHV-1 conserved genes.

(A) Alignment of PCR products sequence with the BHV-1 US7/US8; (B) Alignment of PCR products sequence with the BHV-1 UL49; (C) Alignment of PCR products sequence with the BHV-1 UL48.

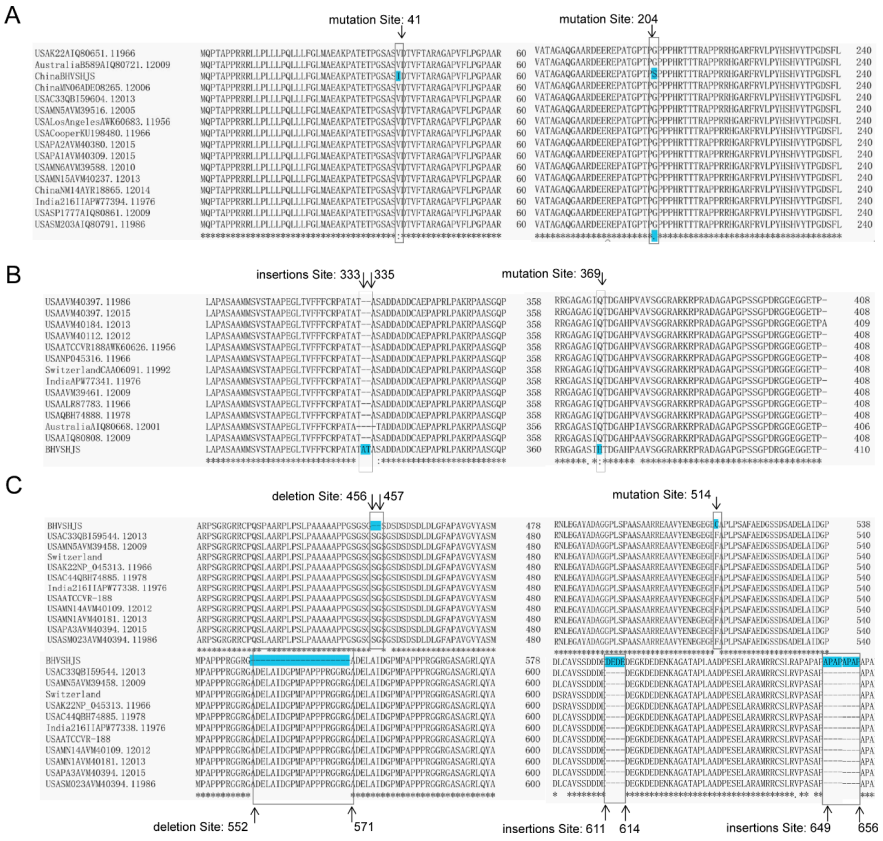


FIGURE S3. Alignment analysis of BHVSHJS US8, UL42 and UL46 amino acid sequence.

(A) Alignment of gE sequence showed that have two mutation site, one site at 41 Val (Val, V) mutated to Ile (Ile, I), the other mutation site at 204 G (Gly, G) mutated to S (Ser, S). (B) Alignment of UL42 sequence showed two insertions at 333 Ser (S) and 334 Ala (A), and one mutation site at 369 H (His) that mutated to Q (Gln). (C) Alignment of UL46 sequence presented two deletion sites at 456 to 457, 552 to 571 and 648

to 653, two insertion regions located at 611 Glu (E) to 614 Asp (D) and 649 Pro (P) to 656 Ala (A), and one mutation site at 514 F (Phe) that mutated to C (Cys).

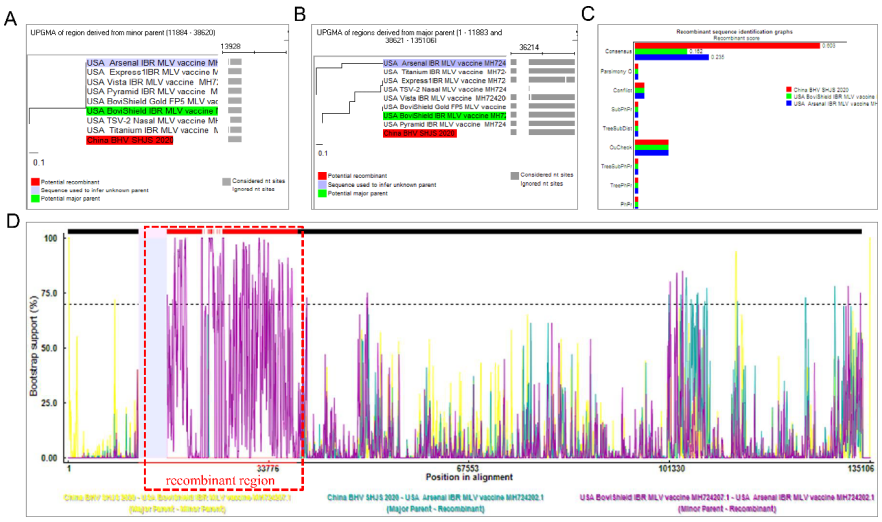


FIGURE S4. The recombinant relationship and Boot-scan analysis of BHVSHJS and vaccine strains.

The tree analysis shown that parents of BHV SHJS were BoviShield IBR MLV and Arsenal IBR MLV vaccine strain on evolutionary relationship of recombinant evens. Recombination score shown that score of BHV SHJS evens was 60.3 %. Boot-scan analysis shown that recombinant region occurred in the BHV SHJS genome at 1,1884 to 38620 position. (A) The tree was constructed based on the sequence of the recombination regions sequences. (B) The tree was constructed based on non-recombinant regions sequences with. (c) Summary of recombination event score on consistency. (C) Recombination events analysis based on Boot-scan method to verify reliability.

A

Genes	Alpha helix (H) % in protein	Extended β strand (E) % in protein	loop (L) % in protein
UL42	17.65	26.72	55.64
UL46	44.74	1.8	53.46

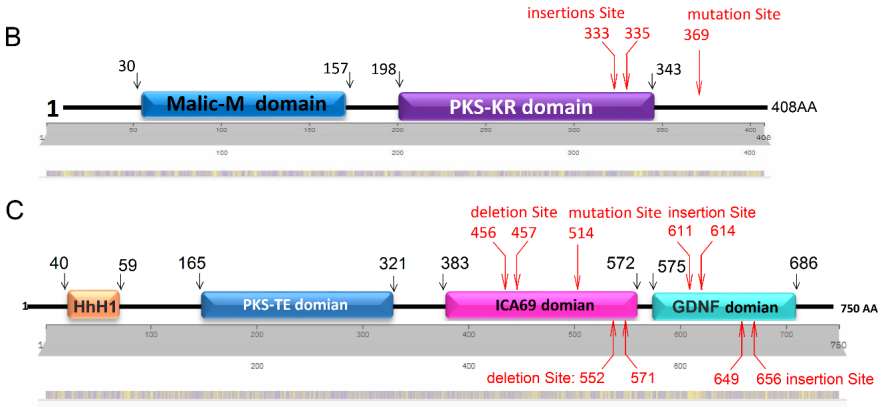
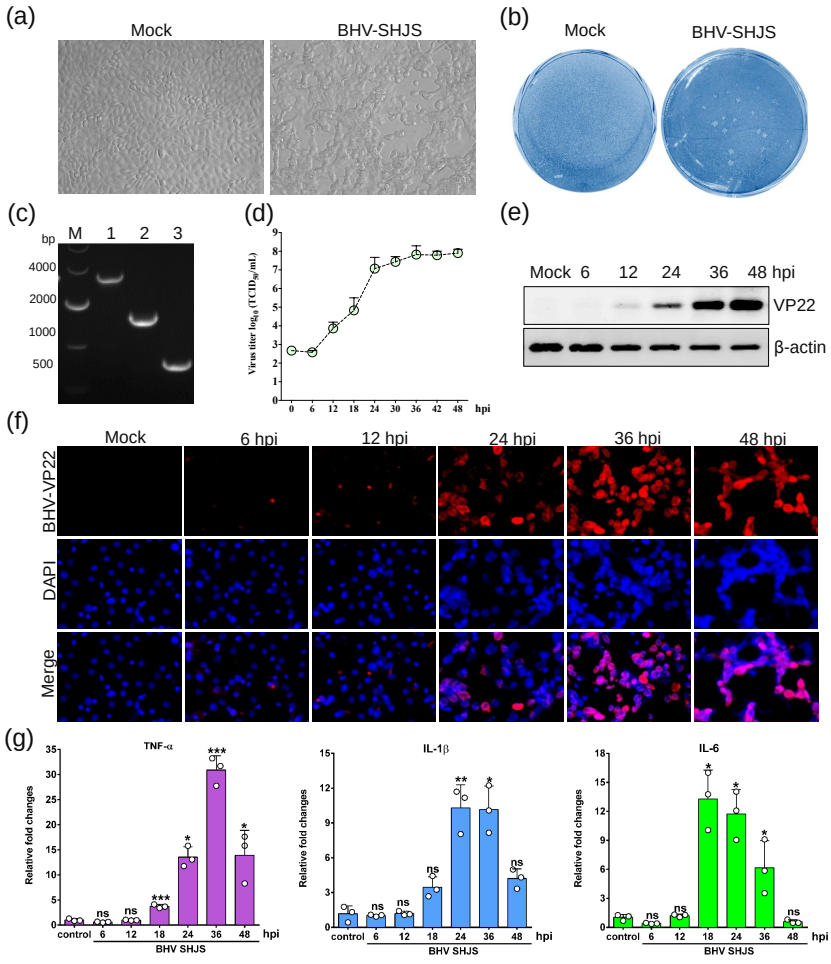
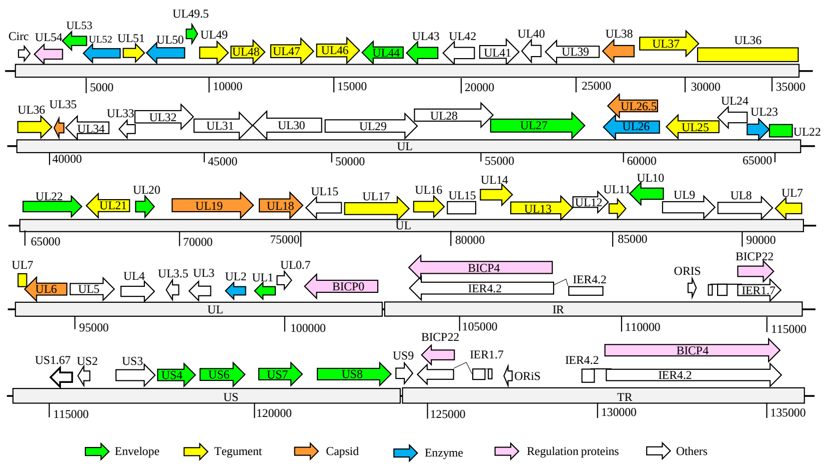


FIGURE S5. Structure of UL42 and UL46 by website prediction.

(A) UL42 and UL46 encoded protein secondary structure were predicted in website( ). (B) UL42 encoded protein domain be was predicted by domain website (http://smart.embl.de/). Result shown that it contains Malic-M, PKS-KR domains. Insertions of BHV SHJS UL42 occurs in the predicted PKS-KR domain. (C) UL46 encoded protein domain be was predicted by domain website (http://smart.embl.de/). Result shown that it contains HhH1, PKS-TE, ICA69 and GDNF domains. Deletions and mutations of BHV SHJS UL46 occurs in an Islet cell autoantigen ICA69 domain and GDNF domain.





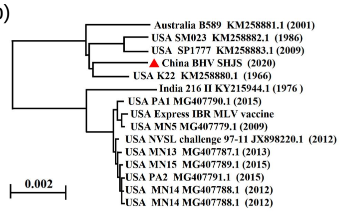
(a)

Gene of mutations, insertions or deletions	UL12	UL19		UL27		UL37					UL42				
Nucleotide position	930	2004	2937	304 or 321	2166	1308	2518	2553	2803	2965 to 2986	369	471	615	980 to 985	1107
China BHV SHJS	A	A	T	G	T	T	T	T	G	GAC~GGG	C	T	A	TGG CGG	G
Australia B589	G	G	C	G	C	C	G	G	A	GAC~GGG	T	C	G	-	T
India 216 II	G	G	C	A	C	C	G	G	A	-	T	C	G	-	T
Switzerland 08-SEP	G	G	C	A	C	C	G	G	A	-	T	C	G	-	T
USA MN14)	G	G	C	A	C	C	G	G	A	-	T	C	G	-	T
USA MN15	G	G	C	A	C	C	G	G	A	-	T	C	G	-	T
USA PA1	G	G	C	A	C	C	G	G	A	-	T	C	G	-	T
USA SM023	G	G	C	G	C	C	G	G	A	-	T	C	G	-	T
USA SP1777	G	G	C	G	C	C	G	G	A	GAC~GGG	T	C	G	-	T

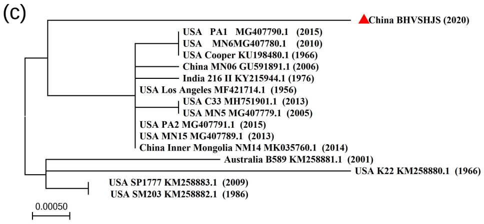
Gene name	UL44		UL46							UL47				US6		US7		US8			
Nucleotide position	1458	1484	822	1369 to 1370	1619 to 1620	1766 to 1777	1775 to 1786	1186	1824	265	558	714	1810	63	1114	306	871	118	348	582 or 610	996
China BHV SHJS	G	C	T	-	-	-	ACG~AGG	C	A	A	G	A	T	G	G	A	C	A	T	A	T
Australia B589	A	A	G	GCT CGG	TGG~CGG	-	-	T	G	A	G	G	C	A	T	A	T	G	C	G	C
India 216 II	A	A	G	GCT CGG	-	-	-	T	G	G	C	G	C	A	T	C	T	G	C	G	C
Switzerland 08-SEP	A	A	G	GCT CGG	-	-	-	T	G	G	C	G	C	A	T	C	T	G	C	G	C
USA MN14)	A	A	G	GCT CGG	TGG~CGG	-	-	T	G	G	C	G	C	A	T	C	T	G	C	G	C
USA MN15	A	A	G	GCT CGG	TGG~CGG	-	-	T	G	G	C	G	C	A	T	C	T	G	C	G	C
USA PA1	A	A	G	GCT CGG	TGG~CGG	-	-	T	G	G	C	G	C	G	G	C	T	G	C	G	C
USA SM023	G	A	G	GCT CGG	-	CGA CGA	ACG~AGG	T	G	G	C	G	C	G	G	C	T	G	C	G	C
USA SP1777	G	A	G	-	-	CGA	ACG~AGG	T	G	G	C	G	C	G	G	C	T	G	C	G	C

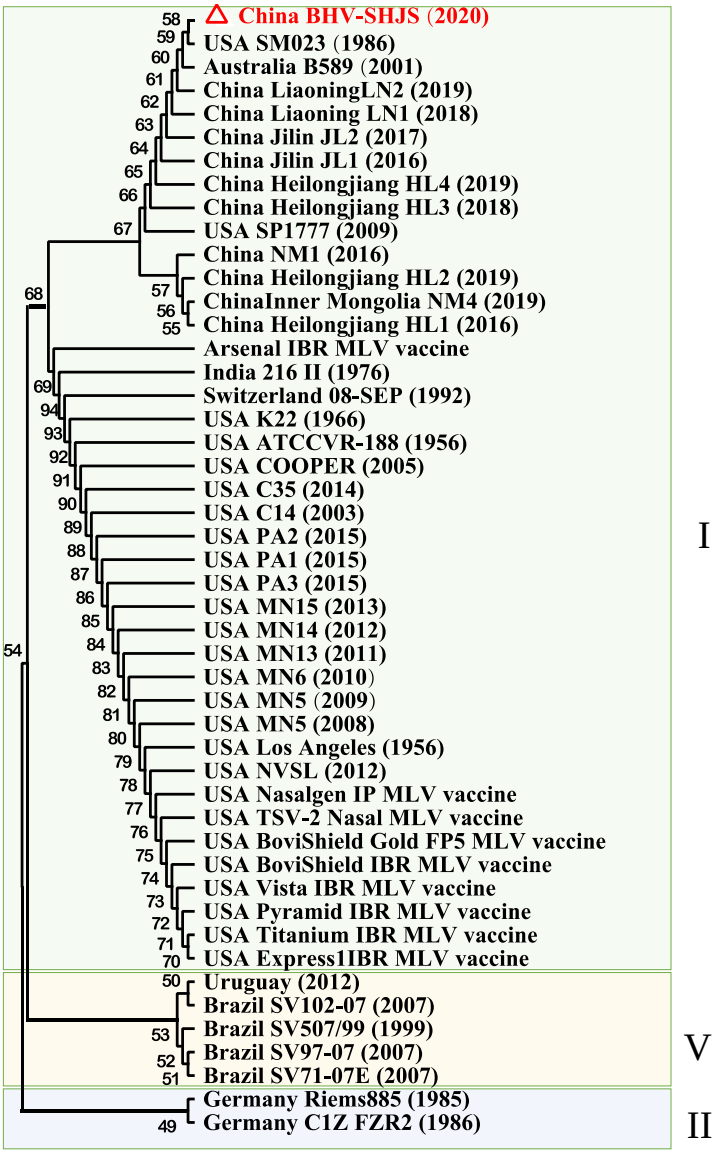
Strain information

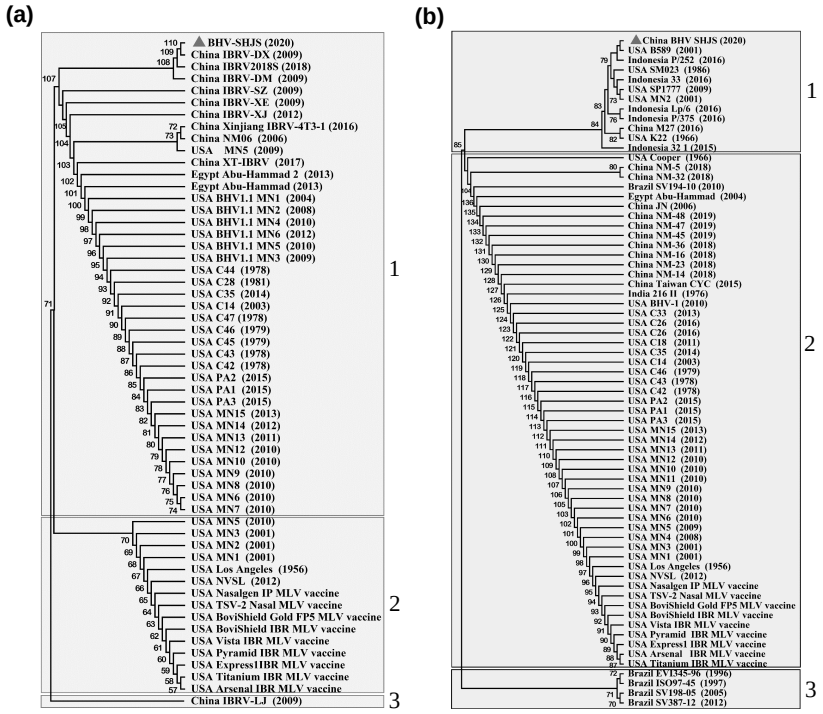
(b)

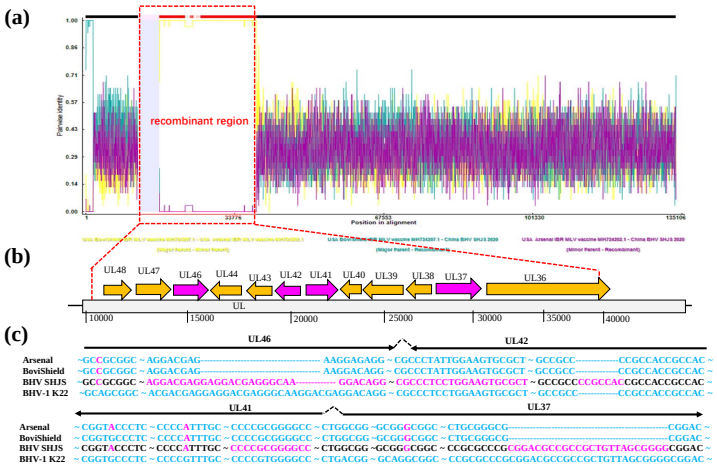


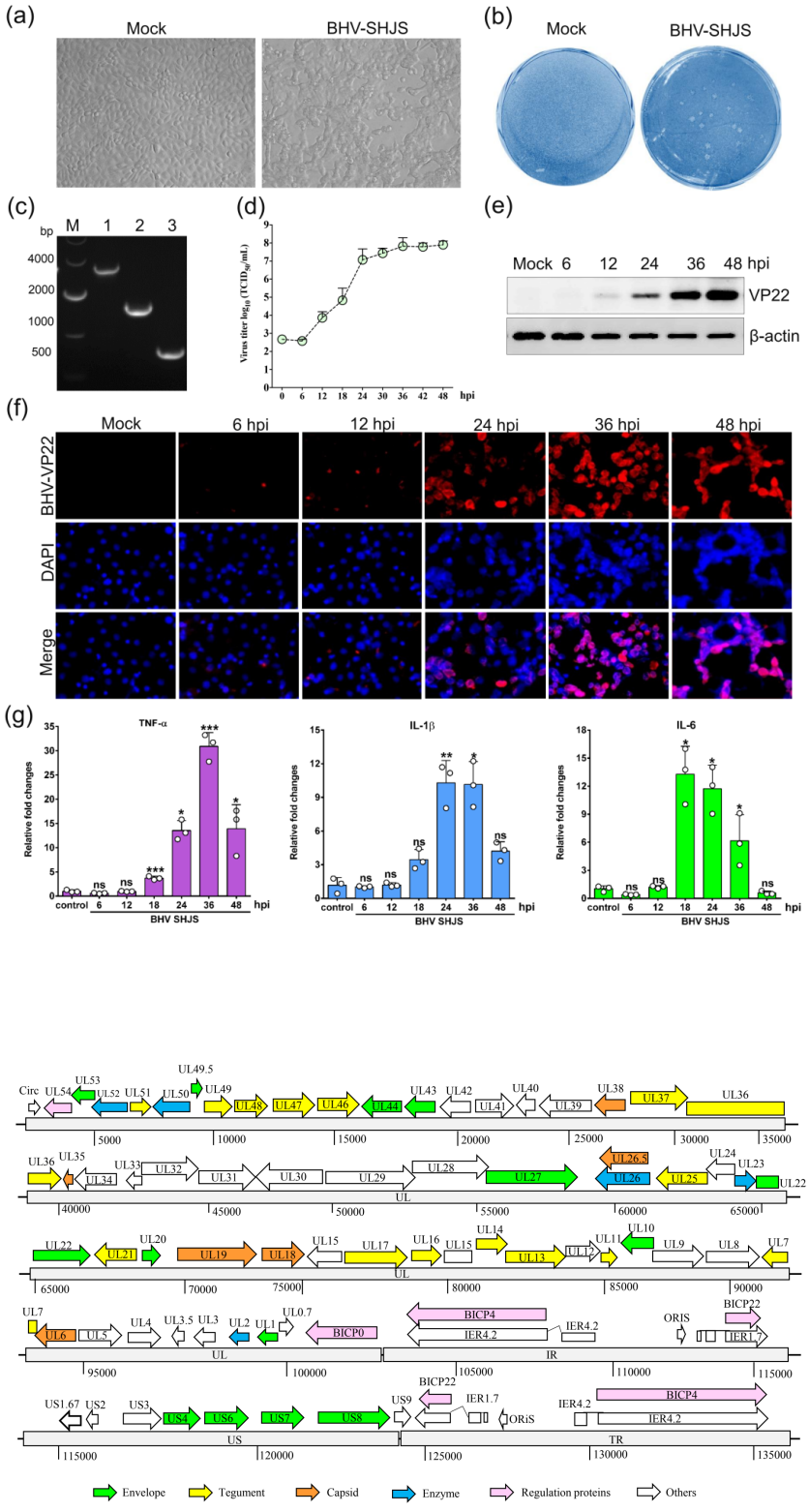
(c)





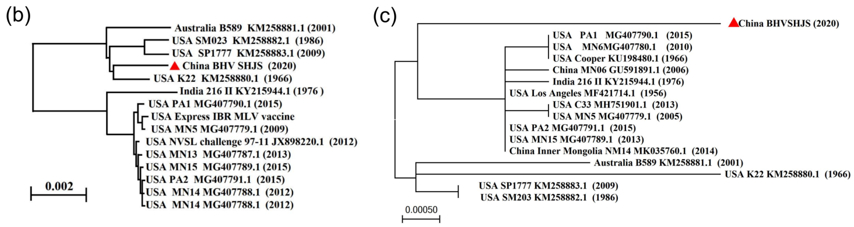


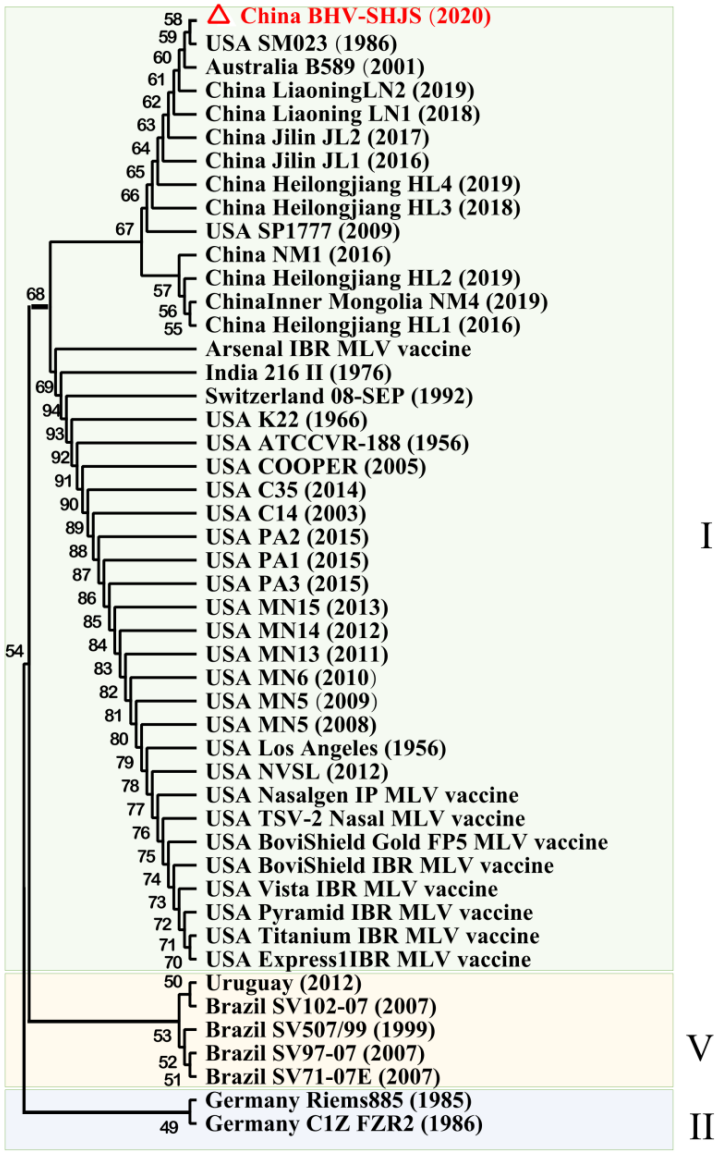


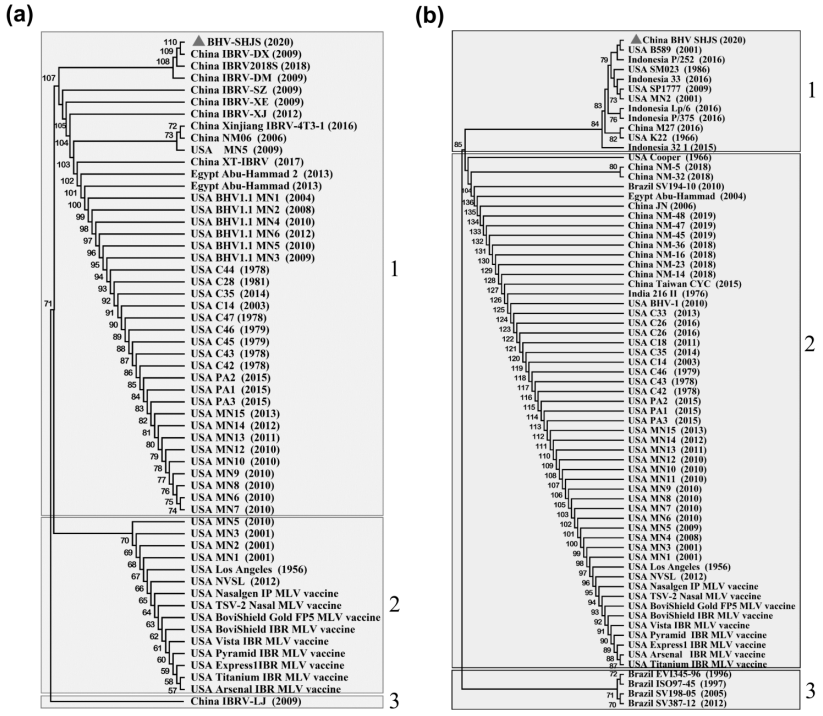


(a)

Gene of mutations, insertions or deletions	UL12	UL19		UL27		UL37					UL42					UL44		UL46							UL47				US6		US7		US8			
Nucleotide position	930	2004	2937	304 or 321	2166	1308	2518	2553	2803	2965 to 2986	369	471	615	980 to 985	1107	1458	1484	822	1369 to 1370	1619 to 1620	1766 to 1777	1775 to 1786	1186	1824	265	558	714	1810	63	1114	306	871	118	348	582 or 610	996
Strain information																																				
China BHV SHJS	A	A	T	G	T	T	T	T	G	GAC~ GGG	C	T	A	TGG CGG	G	G	C	T	-	-	-	ACG~ AGG	C	A	A	G	A	T	G	G	A	C	A	T	A	T
Australia B589	G	G	C	G	C	C	G	G	A	GAC~ GGG	T	C	G	-	T	A	A	G	GCT CGG	TGG~ CGG	-	-	T	G	A	G	G	C	A	T	A	T	G	C	G	C
India 216 II	G	G	C	A	C	C	G	G	A	-	T	C	G	-	T	A	A	G	GCT CGG	-	-	-	T	G	G	C	G	C	A	T	C	T	G	C	G	C
Switzerland 08-SEP	G	G	C	A	C	C	G	G	A	-	T	C	G	-	T	A	A	G	GCT CGG	-	-	-	T	G	G	C	G	C	A	T	C	T	G	C	G	C
USA MN14)	G	G	C	A	C	C	G	G	A	-	T	C	G	-	T	A	A	G	GCT CGG	TGG~ CGG	-	-	T	G	G	C	G	C	A	T	C	T	G	C	G	C
USA MN15	G	G	C	A	C	C	G	G	A	-	T	C	G	-	T	A	A	G	GCT CGG	TGG~ CGG	-	-	T	G	G	C	G	C	A	T	C	T	G	C	G	C
USA PA1	G	G	C	A	C	C	G	G	A	-	T	C	G	-	T	A	A	G	GCT CGG	TGG~ CGG	-	-	T	G	G	C	G	C	G	G	C	T	G	C	G	C
USA SM023	G	G	C	G	C	C	G	G	A	-	T	C	G	-	T	G	A	G	GCT CGG	-	CGA CGA	ACG~ AGG	T	G	G	C	G	C	G	G	C	T	G	C	G	C
USA SP1777	G	G	C	G	C	C	G	G	A	GAC~ GGG	T	C	G	-	T	G	A	G	-	-	CGA	ACG~ AGG	T	G	G	C	G	C	G	G	C	T	G	C	G	C







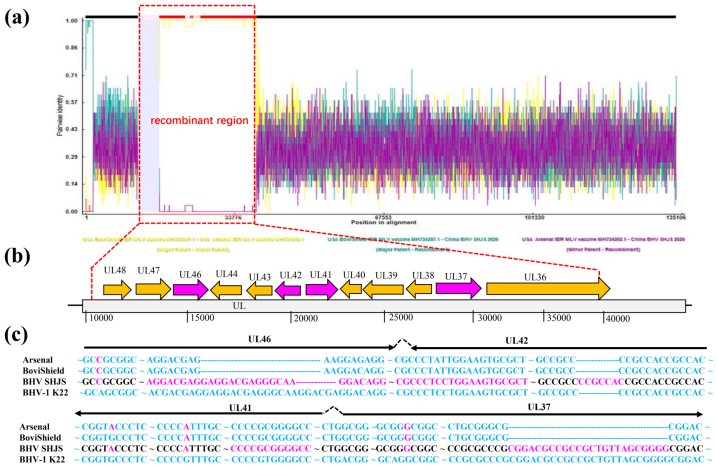


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Table 1.docx available at https://authorea.com/users/497461/articles/578493-the-first-full-genome-characterization-and-phylogenetic-analysis-of-a-potential-recombinant-bovine-herpesvirus-type-1-isolated-in-china