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Prevalence, Genome Characterization, and Phylogenetic Analysis of Bovine Hepacivirus in Inner Mongolia, Northeastern China

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Abstract

Objective: Bovine hepacivirus (BovHepV) is a new member of the genus *Hepacivirus* in the family *Flaviviridae*, which has been detected in cattle in more than seven countries. The purpose of the study was to identify and genetically characterize BovHepV in cattle in northeastern China.

Methods: A total of 116 serum samples from free-range cattle were collected from HulunBuir city in April and May, 2021, and were divided into three pools before being subjected to metagenomic sequencing. The BovHepV sequences obtained were used for genome characterization and phylogenetic analysis.

Results: Two 8840 nucleotides long BovHepV strains YKS01/02 were identified in the samples through metagenomic sequencing. In RT-PCR screening, the prevalence of BovHepV was 8.8% (5/57) in Yakeshi of HulunBuir. The novel virus showed a range of 79.3%–91.9% nucleotide sequence identity and 92.5%–98.1% amino acid sequence identity with the BovHepV sequences discovered. Phylogenetic analysis classified YKS01/02 strains into BovHepV subtype G strains discovered in Jiangsu Province, China, and named with subtype G2.

Conclusion: This study first identified BovHepV in cattle in northeastern China, and expands the geographical distribution and genetic diversity of BovHepV in the country.

Keywords: Bovine hepacivirus; Phylogenetic analysis; Metagenomic sequencing; Northeastern China; Cattle

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INTRODUCTION

The genus *Hepacivirus*, belonging to the family *Flaviviridae*, comprises 14 genetically diverse viral species (*Hepacivirus* A–N) that infect humans and a wide variety of animals, including rodents, bats, shrews, dogs, equines, cattle, monkeys, and even birds and aquatic animals [1-10]. Bovine hepacivirus (BovHepV) is a newly identified Hepatitis C virus (HCV)-like virus identified in cattle [11, 12]. Like other hepaciviruses, BovHepV has a single-stranded positive-sense RNA genome with ~8.8 kb nucleotide in length, which encodes a single polyprotein and can be cleaved into structural proteins (Core, E1, and E2) and nonstructural proteins (p7, NS2, NS3, NS4a, NS4b, NS5a, and NS5b) by proteases from the host and virus [6, 13].

BovHepV was discovered in German dairy cow serum using high-throughput sequencing technology in 2015 for the first time, and thereafter listed as the Hepacivirus N species. The virus has been detected in more than seven countries distributed in five continents, with viral RNA prevalence ranging from 0.6% to 14.8% [6, 11-17]. In China, four BovHepV subtypes (three in genotype 1 and one in genotype 2) have already been confirmed in the Guangdong, Yunnan, Jiangsu, and Sichuan Provinces, suggesting a complex genetic diversity and an extensive geographic distribution of the virus [11, 12, 14, 18-20]. It is remarkably, however, that the BovHepV had been detected in the ticks collected from cattle in Guangdong Province, which indicate that ticks may play an important role in the transmission of the virus.

To date, the risk of BovHepV on public health is still unclear, and the potential zoonotic transmission risk also needs to be further confirmed. Therefore, it is necessary to make clear the epidemic distribution of the virus worldwide, which may help to monitor the high-risk groups (*e.g.*, cattle husbandry or slaughter workers) in BovHepV positive areas. To our knowledge, no epidemiologic study on BovHepV has been conducted in northeastern China. In this study, we identified and genetically characterized BovHepV in cattle in HulunBuir, Inner Mongolia, northeastern China, which expands the geographical distribution and genetic diversity of BovHepV in the country, providing useful information on the prevalence and genetic characteristics of the virus.

METHODS

Sample collection

Hulunbuir (115°31'–126°04' E and 47°05'–53°20' N) is one of the biggest prefectural-level cities of the Inner Mongolia Autonomous Region in northeastern China, which is the boundary zone of Mongolia, Russia, and China, and is famous for its prairie and animal husbandry with a number of more than one million cattle and eight million sheep (http://tjj.hlbe.gov.cn/index/acticle/show/id/643.html). In this study, blood samples of free-range cattle were collected from Ergun, Genhe, and Yakeshi in HulunBuir from April to May, 2021. Serum was separated by centrifugation at 500 rpm/min for 10 min and stored at −80°C until use.

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RNA library construction and metagenomic sequencing

A total of 116 free-range cattle serum samples were collected from HulunBuir in Inner Mongolia, northeastern China. The samples were divided equally into three pools, and used for RNA library construction. After being digested with micrococcal nuclease (NEB, USA) in 37°C for two hours, the pooled serum samples were used for RNA extraction using TIANamp Virus RNA Kit (TIANGEN, China) according to the manufacturer's instructions and sent for metagenomic sequencing. The Illumina sequencing and library construction were performed at Tanpu Biological Technology Co., LTD (Shanghai, China). Briefly, the RNA from each pool was used for library preparation with the NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) according to the manufacturer's instructions. After adapter ligation, ten cycles of PCR amplification were performed for sequencing target enrichment. The libraries were pooled at equal molar ratio, denatured and diluted to optimal concentration, and sequenced with an Illumina NovaSeq 6000 System.

Transcriptome analysis and virus discovery

Downstream bioinformatics analysis of raw data was conducted as described elsewhere [21]. Briefly, after trimming and removing low quality reads, the paired-raw reads were further purified by removing ribosomal RNA, host contamination, and bacteria sequences using BBMap program (https://github.com/BioInfoTools/bbmap), and assembled into contigs with SPAdes v3.14.1 (https://github.com/abl ab/spades) and SOAPdenovo v2.04 (https://github.com/aquaskyline/SOAPdenovo-Trans) [22, 23]. After being compared with the non-redundant nucleotide (nt) and protein (nr) database downloaded from GenBank using BLAST+ v2.10.0, the assembled contigs were further filtered to remove the host and bacterial sequences. The relative abundance of the identified viruses was determined by mapping the reads back to the assembled contigs using Bowtie2 v2.3.3.1.

Virus detection and complete genome amplification

To verify the results of metagenomic sequencing, semi-nested RT-PCR with primers designed based on the BovHepV sequences obtained from metagenomic sequencing were used to identify the virus in all the 116 samples. Viral RNA of serum samples was extracted using a TIANamp Virus RNA kit (TIANGEN, China) and reversed using a PrimeScript 1st Strand cDNA Synthesis kit (TaKaRa, Japan) in accordance with the manufacturer's instructions. The specific primers of F (5′-GGCTCACCYTCACATGATCCCT-3′) and R1 (5′-ACCAAGGACATGATTCCGCAAA-3′) for the first round, and F and R2 (5′-TGGAACCATTCGGCTTAACACT-3′) for the second round were used for BovHepV detection. The PCR amplification system was as follows, briefly, the 25-μL PCR reaction mixture comprised 12.5 μL of Premix Taq (TaKaRa), 1 μL of F and R1/R2 primers for each, 1 μL of template cDNA, and 9.5 μL ddH_2O. Amplification parameters were

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94°C for 5 min followed by 35 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. To avoid cross-contamination, we carried out the following actions as described elsewhere [24], namely, (i) separated the pre- and post-PCR areas physically, (ii) cleaned the work surfaces and equipment with 1% (vol/vol) sodium hypochlorite solution, (iii) decontaminated the work surfaces and equipment by UV exposure, (iv) set up negative controls to verify cross-contamination. Moreover, semi-nested PCR were also used to amplify the complete genome sequences of BovHepV from positive samples using the primers in Table S1.

Sequence comparison and phylogenetic analysis

The open reading frames were predicted by ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder) and annotated as previously described [12]. BovHepV sequences were retrieved from GenBank database, and the downloaded nucleotide (nt) and amino acid (aa) sequences were aligned using ClustalW and calculated for identities by MegAlign program available within DNAstar V7.1. The phylogenetic relationships were estimated using the maximum likelihood method conducted in MEGA version 7.0 [25] with default settings. We conducted a bootstrapping analysis of 1000 replicates. The bootstrap values > 70% were considered significant and shown in the trees.

Recombination analysis

Recombination events of BovHepV were detected using the RDP4 software package, which integrates seven recombination detection methods [26]. The analyses were performed with default settings, and recombination events should satisfy the following two conditions according to the RDP manual's suggestion, namely, (i) there should be at least two methods showing P value < 0.05; and (ii) the RDP recombination consensus score (RDPRCS) should be > 0.60. If the recombination event met the first condition, but RDPRCS was between 0.4 and 0.6, a possible recombination event can be considered. Otherwise, the event was considered untenable. Additionally, to further confirm the recombination events, the aligned BovHepV sequences were also analyzed using Simplot version 3.5.1 [19].

RESULTS

Sample collection and BovHepV identification

A total of 116 free-range cattle serum samples were collected from Yakeshi (57), Ergun (40), and Genhe (19) of HulunBuir in April and May, 2021(Figure 1). After metagenomic sequencing, two pooled BovHepV positive samples were sequenced. A total of 1259 reads were annotated as sequences of BovHepV/JS/02 with a mean depth of 91.8 (Figure 2), and assembled into two 8840-length BovHepV sequences, showing a nucleotide sequence identity of 91.9% and an amino acid sequence identity of 98.1% with BovHepV subtype G strain JS05. To verify the results of metagenomic sequencing, semi-nested RT-PCR with primers designed based on the BovHepV sequences obtained from metagenomic sequencing were used to determine the virus in all the 116

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samples. Five serum samples from Yakeshi were identified BovHepV positive, showing a prevalence of 8.8% (Figure 1). Considering the high nucleotide identity (100%) of the sequences obtained from the five samples using the Sanger Sequencing method, we only amplified the complete genome sequences from two samples with the designed primers (Table S1) using semi-nested RT-PCR, and the assembled BovHepV sequences were designated as YKS01 and YKS02 with GenBank accession numbers OM131409 and OM131410.

Viral genome analysis

The genomes of BovHepV/YKS01/02 strains were 8840 nucleotides long, and had an 8340-nucleotide-long polyprotein gene, with a genome organization of 5′ UTR-core-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3′ UTR (Figure 3A).

Similarity analysis showed that BovHepV/YKS01/02 strains shared 100% sequence identity in both nucleotide and amino acid level, while the two strains showed a range of 79.3%–91.9% nucleotide sequence identity and 92.5%–98.1% amino acid sequence identity with the discovered BovHepV sequences (Table 1). Moreover, the identified strains had a nucleotide identity of 91.5%–91.9% with subtype G strains isolated from Jiangsu Province, China, but had a lower nucleotide identity of 79.3%–84.7% with other subtype strains from Ghana, Germany, Brazil, and Guangdong Province, China (Table 1). Considering the criteria that a novel subtype of HCV is confirmed if it shows a nucleotide sequence identity of < 85% with other strains [27], we concluded that the identified strains belong to subtype G BovHepV.

SimPlot analysis showed the changing trend of polyprotein amino acid similarity among the eight BovHepV subtypes (Figure 3). Notably, the sequences of identified strains in this study had a lower amino acid similarity in NS5A protein, showing a low amino acid similarity of 81%–90% with that of B, C, E, and F BovHepV subtypes (Figure 3B).

Phylogenetic analysis and recombination analysis

The phylogenetic analysis results based on the amino acid and nucleotide sequences of complete polyprotein of BovHepV subtypes A–H including the newly identified sequences in this study were similar in topology (Figure 4). The BovHepV strains were clearly separated into eight branches. The novel identified strains were corresponding to the BovHepV subtype G isolates but located in a different branch (Figure 4). Considering the different topology and nucleotide sequence identity (91.5–91.9%), we classified the novel isolates as subtype G2 to distinguish between other subtype G strains discovered in Jiangsu Province, China. The result of phylogenetic analysis was consistent with the identity analysis showed in Table 1. Moreover, recombination analysis based on RDP4 showed no potential recombination events among YKS01/02 strains and

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other BovHepV strains.

Discussion

Since it was reported in Ghana and Germany in 2015, bovine hepacivirus (BovHepV) has been identified in at least seven countries distributed in five continents [6, 11, 13-18, 20], and has been classified into two genotypes and eight subtypes (belong to genotype 1) [12], suggesting that the virus may have a wider geographical distribution and genetic diversity. In China, BovHepV has been detected in southern and southwestern provinces, including Guangdong, Jiangsu, Yunnan, and Sichuan Province. To date, no epidemiologic study about BovHepV has been conducted in north or northeastern China, which includes many livestock farming provinces (*e.g.,* Inner Mongolia, Jilin, and Heilongjiang Province). In this study, we identified subtype G BovHepV in HulunBuir, Inner Mongolia, northeastern China (Figure 1, Figure 4), which confirmed the presence of BovHepV in cattle in northeastern China for the first time, and expanded the virus distribution areas in the country.

There are at least four BovHepV subtypes (three in genotype 1 and one in genotype 2) circulating in China [11, 12, 14, 18, 20]. Notably, other BovHepV prevalent countries, such as Germany and Italy, were also shown to have multiple genotypes or subtypes co-circulating, suggesting a complex BovHepV genotypes and subtypes geographic distribution worldwide. Although the novel identified BovHepV strains YKS01/02 were classified into subtype G, the different topology and low nucleotide sequence identity (91.5%–91.9%) of the virus compared with other subtype G strains further indicated the genome diversity of BovHepV in the same subtype.

Except the 5′ UTR as described elsewhere was conserved among the different BovHepV strains [11], our study indicated that the Core, p7, NS4A, NS4B, and NS5B proteins among different BovHepV subtypes showed an amino acid identity of >95% (Figure 3), which suggested that these protein genes are relatively conserved and can be used as the target region for BovHepV nucleic acid detection. In contrast, NS5A protein showed relative low identities between different genotypes, and was recommended to be used target protein gene for genotyping of BovHepV. As a novel hepacivirus species identified in recent years, the function and pathogenesis on the proteins of BovHepV are still unclear, but the mechanism studies on HCV have made great achievements, which will provide references for BovHepV study. For instance, studies have confirmed that NS5A of HCV is a multifunctional protein that associate with viral RNA replication and host cellular signaling pathways regulation[28-30]. It also has been verified that the targeted inhibitors of NS5A have good efficacy in the treatment of HCV[31].

As a newly identified HCV-like viruses, there are many epidemiological issues that need further investigation. Firstly, more epidemiological studies should be conducted on different cattle species and in more cattle husbandry countries. Secondly, we need to know whether BovHepV can cause hepatitis or other diseases in cattle, since HCV is a human pathogen and can cause human

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hepatitis and liver failure. It is worth mentioning that the BovHepV RNA positive cattle in our study did not show any clinical symptoms. Moreover, one more important thing is to know whether the BovHepV is a zoonoses pathogen. To determine the pathogenicity of BovHepV on human beings, we consider that serological or molecular biological detection should be done in cattle husbandry and slaughter workers in BovHepV positive areas. Lastly, as BovHepV strains have been detected in blood-sucking ticks collected from cattle [12], we suggest that more questing ticks in the BovHepV prevalent areas should be collected and screened for the virus, and the vector competence of ticks in transmitting BovHepV also should be validated if possible. It is remarkably, however, that commercial fetal bovine serum has been confirmed the presence of BovHepV [11]. As one of the most widely used animal-derived biological reagents for cell culture, bovine serum contaminated with viral may pose serious adverse impacts to experimental results. Therefore, it is necessary to detect BovHepV in donor cattle and eliminate the positive groups, in addition, BovHepV positive commercial fetal bovine serum should also be removed before cell cultures.

There are some limitations in the present study. Firstly, limited bovine serum samples and collection sites were involved in this study, which may not present the prevalence and distribution of BovHepV in northeastern China objectively. Moreover, we didn't try to isolate the virus from positive samples, which may limit the further study on virulence and pathogenicity of virus. Lastly, ticks in the surveyed sites including blood-sucking ticks from cattle and questing ticks were not collected for BovHepV detection, and the role of ticks in the transmission of BovHepV in northeastern China was unclear.

CONCLUSION

In conclusion, BovHepV RNA was detected in cattle in HulunBuir, Inner Mongolia, northeastern China for the first time, showing a prevalence of 8.8% in the collection site. The findings expand the geographical distribution and the genetic diversity of BovHepV in China, and provide useful information regarding the prevalence and genetic characteristics of the virus.

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COMPETING INTERESTS

The authors declare that there are no conflicts of interest.

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ETHICAL APPROVAL

This study was approved by the Animal Administration and Ethics Committee of the First Hospital of Jilin University. All the cattle, from which the serum samples were collected, were handled humanely, and all procedures were performed strictly in accordance with the requirements of Animal Ethics Procedures and Guidelines of the People's Republic of China.

AUTHOR CONTRIBUTIONS

Zedong Wang, Quan Liu, Feng Wei, and Xianmin Feng designed the study. Yu Gu, Liang Li, and Wei Wang collected the cattle serum samples. Ziyan Liu, Wenbo Xu, Yongxu Yuan, Xiaojie Liang, Yinghua Zhao, and Liyan Sui conducted the molecular tests and bioinformatics analysis. Ziyan Liu, Zedong Wang, and Quan Liu wrote the report. All authors contributed to review and revision and have seen and approved the final version.

DATA AVAILABILITY STATEMENT

The data is available with the corresponding author and will be produced on request.

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Table1. Sequence identity of BovHepV polyprotein gene at the nucleotide (upper right) and amino acid (lower left) levels ca
available within DNAstar V7.1. The compared strains were indicated as accession number and country, and the different colo
subtypes/genotypes of the BovHepV strains.

Strains		Subtype A						Subtype B			Subtype C		Subtype D		Subtype E		Subtype F
		A1	A2	A3	A4	A5	A6	B1	B2	B3	C1	C2	D1	D2	E1	E2	F1
A1	KP641123__BovHepV_B1Ger2013		93.3	93.8	91	93.6	90.8	80.1	79.8	79.9	80.3	80.4	82.4	82.5	79.5	79.5	80.1
A2	KP641124_BovHepV_209Ger2014	97.9		93.1	91.6	93.5	90.9	79.9	79.8	80	80.5	80.7	82.5	82.5	79.5	79.5	80.1
A3	KP641125_BovHepV_379Ger2014	98.2	98.1		91.4	93.6	91	80.2	79.8	80	80.3	80.5	82.5	82.8	79.5	79.5	80.1
A4	KP641126_BovHepV_438Ger2014	97.5	97.4	97.4		91.7	90.5	79.6	79.7	80.1	80.2	80.3	82.2	82.3	78.9	78.9	79.6
A5	KP641127_BovHepV_463Ger2014	98.1	98.1	98	97.5		90.9	80	79.9	80.2	80.3	80.6	82.5	82.9	79.6	79.6	80.3
A6	MH027953_BovHepV_BH204/16-6	97.3	97.4	97.2	97.1	97.6		79.5	79.7	79.8	79.8	80.1	82.3	82.5	79.5	79.5	79.6
B1	KP265950_BovHepV_GHC100	92.9	93.1	93.3	92.7	93	93		90.4	90.9	82.7	82.8	80.7	80.5	82	82	81.6
B2	KP265948_BovHepV_GHC85	93.1	93.3	93.4	92.8	93.2	93.1	98.7		92	82.4	82.7	80.2	80.5	81.7	81.7	81.1
B3	KP265943_BovHepV_GHC25	93	93	93.1	92.8	93.1	93	98.7	99		82.7	82.9	80.4	80.7	81.7	81.7	81.4
C1	KP265946_BovHepV_GHC52	92.3	92.4	92.5	92.3	92.3	92	95.5	95.5	95.6		98.4	80.9	80.9	81.5	81.4	81.3
C2	KP265947_BovHepV_GHC55	93	93.1	93.2	92.9	93.1	92.7	96.2	96.3	96.3	98.8		81.1	81.1	81.6	81.6	81.5
D1	MG781018_Hepacivirus_N_BR_MA236B017	93.8	94.1	94.4	93.9	93.9	94.3	93.5	93.3	93.1	92.7	93.4		93.9	80.2	80.2	80
D2	MG781019_Hepacivirus_N_BR_RN034B019	93.6	93.9	94.3	93.8	93.7	94	93.5	93.4	93.2	92.5	93.2	98.1		80.7	80.7	80
E1	MG257793_BovHepV_GD/01	92.1	92.1	92.2	91.7	92.1	92	95.4	95.3	95.3	94.4	94.8	92.7	92.6		99.8	81
E2	MG257794_BovHepV_GD/02	92.1	92.1	92.3	91.7	92.2	92.1	95.4	95.3	95.4	94.5	94.9	92.7	92.6	100		81
F1	MH027948_BovHepV_	92	92.1	92.3	92	92.2	92.2	95.2	94.8	94.8	94.4	95	92.6	92.9	94.3	94.4	
G1	OM131409 Bovine hepacivirus YKS01	95.6	96	96.1	95.9	96	96.1	93.6	93.8	93.5	92.9	93.5	94.9	94.9	92.5	92.5	92.7
G2	OM131410 Bovine hepacivirus YKS02	95.6	96	96.1	95.9	96	96.1	93.6	93.8	93.5	92.9	93.5	94.9	94.9	92.5	92.5	92.7
G3	MN266283_BovHepV_JS02	95.3	95.6	95.7	95.6	95.7	95.8	93.4	93.4	93.2	92.7	93.2	94.7	94.7	92.5	92.5	92.5

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G4	MN266284_BovHepV_JS05	95.4	95.6	95.6	95.5	95.7	95.6	92.9	93.1	92.8	92.4	92.9	94.5	94.4	92.3	92.3	92.2
G5	MN266285_BovHepV_JS06	95.3	95.5	95.5	95.3	95.6	95.5	92.8	93	92.7	92.4	92.9	94.4	94.3	92.3	92.3	92.1
H1	MZ540979_BovHepV_GDZJ02-2	94	94.1	94.4	94	93.9	94.1	92.8	92.9	92.7	92.1	92.8	95.6	95.5	92	92.1	92.2
H2	MZ221927_BovHepV_GDZJ02-1	94	94.1	94.4	94	93.9	94.1	92.8	92.9	92.7	92.1	92.8	95.6	95.5	92	92.1	92.2
H3	MZ540980_BovHepV_GDZJ02-3	94	94.1	94.4	94	93.9	94.1	92.8	92.9	92.7	92.1	92.8	95.6	95.5	92	92.1	92.2

DOI: 10.55415/deep-2022-0007.v1

Figure legends

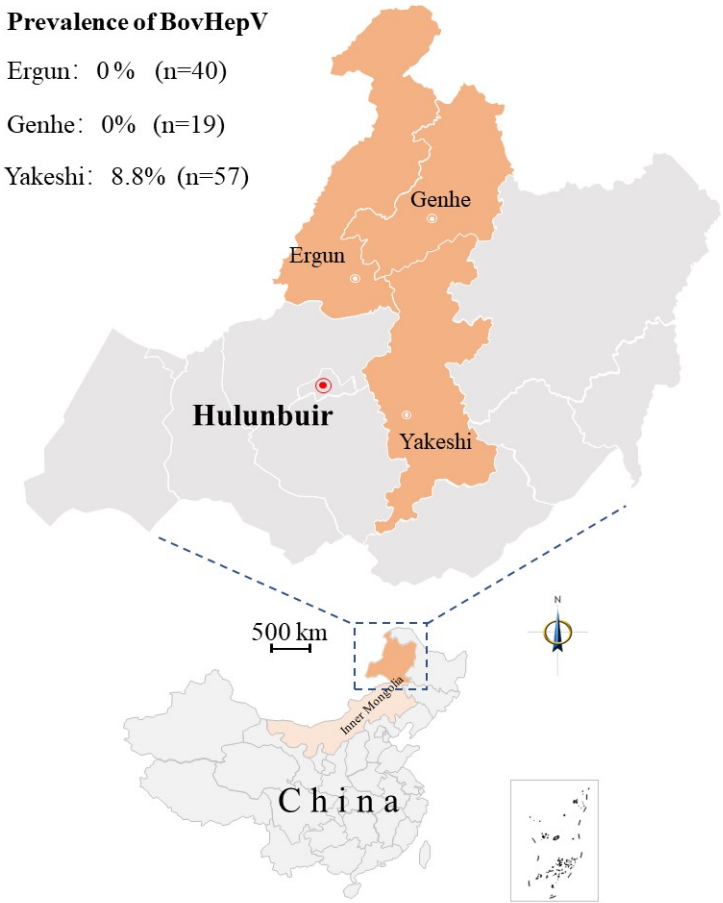

Figure 1. Sampling sites of cattle serum samples for the study in Hulunbuir, northeastern China. The orange shadowed areas represent the sampling sites.

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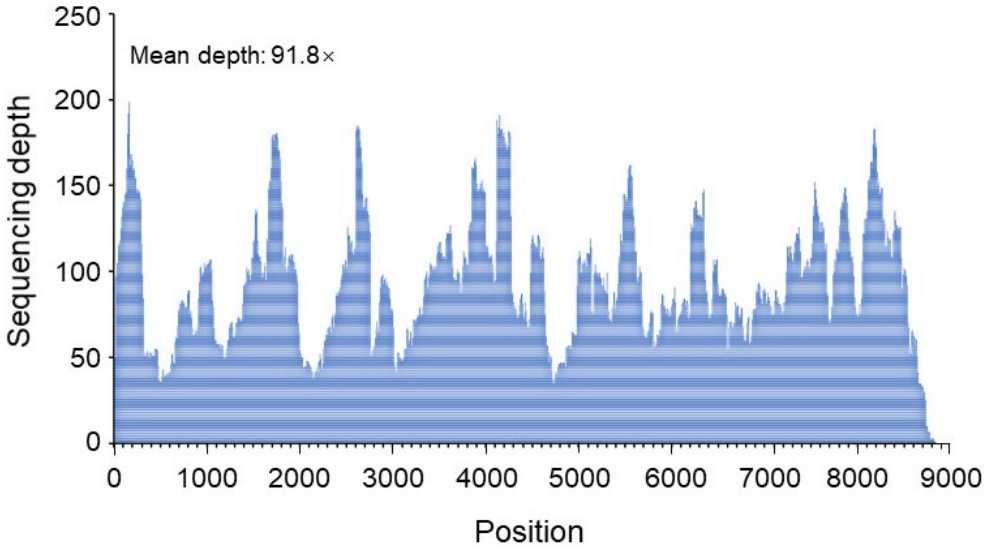

Figure 2. Mapped read count plot of the sequenced BovHepV genome. The histograms show the depth of coverage for each base of the viral genome. The mean depth of the virus genome was 91.8×.

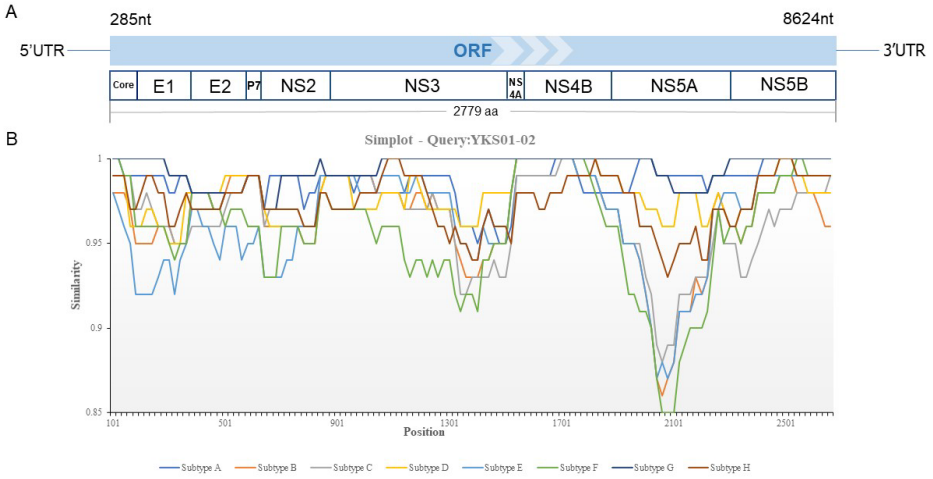

Figure 3. Genomic characterization and SimPlot analysis of BovHepV based on amino acid of polyprotein. A. Genome organization of BovHepV identified in this study. B. Simplot analysis of BovHepV subtypes A–H. The identified strains BovHepV/YKS01/02 were used as the query strains, and the analysis was calculated using Simplot version 3.5.1 with a sliding window of 200 and a step size of 20 residues. Different colors indicate different subtypes of BovHepV. The strain names and GenBank accession numbers of the BovHepV strains are listed in Table S2.

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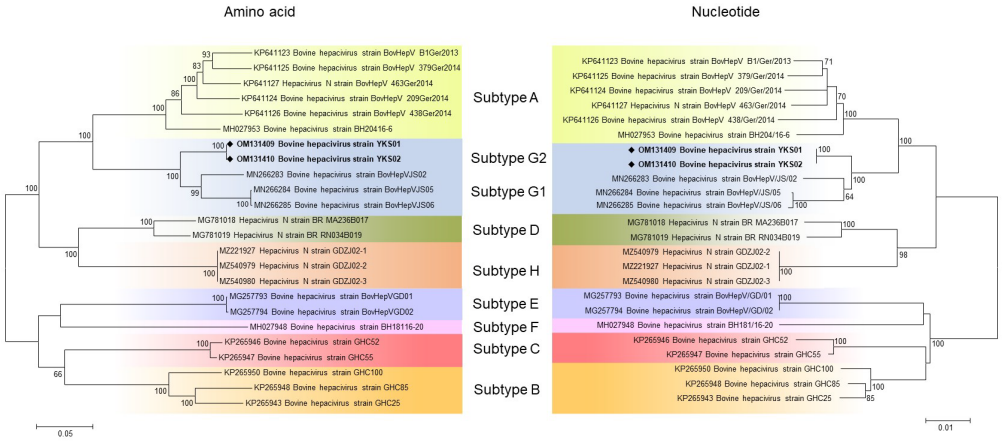

Figure 4. Phylogenetic analysis based on the amino acid and nucleotide sequences of the complete polyprotein of BovHepV subtypes A–H including the newly identified sequences in this study. The trees were constructed based on the maximum likelihood method using MEGA 7.0 with default settings. A bootstrapping analysis of 1000 replicates was conducted, and the bootstrap values > 70% were considered significant and shown in the trees. GenBank accession numbers were shown, followed by the name of the virus strains. Different colors indicated different subtypes of BovHepV, and black rhombus represented the BovHepV identified in this study.