



# Detection, genetic, and codon usage bias analyses of the VP2 gene of mink bocavirus

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## Abstract

Mink bocavirus 1 (MiBoV1), a novel virus detected from the feces of domestic minks in China in 2016, may be related to gastrointestinal diseases. However, its prevalence and genetic characteristics are poorly described. In this study, we examined 192 samples collected from minks in the major mink industry province from northern China. PCR results showed that 10 samples (5.2%) were positive for MiBoV1, and 60% of MiBoV1-positive samples were co-infected with Aleutian mink disease virus or mink circovirus. MiBoV1 was detected in six serum samples. Sequence analysis demonstrated that the VP2 gene of MiBoV1 was highly conserved and had low viral diversity over the VP2 region and unique nucleotide mutations. Phylogenetic analysis of the VP2 sequence demonstrated that MiBoV1 strains formed two clades and were grouped with California sea lion bocavirus, Canine bocavirus, and Feline bocavirus. Codon usage analysis revealed that most of the preferentially used codons in MiBoV1 were A- or U-ended codons, and no evident codon usage bias was found. This study provides evidence that MiBoV1 has a low prevalence in Jilin and Hebei provinces in China. Moreover, it contributes information regarding the expansion of the limited mink bocavirus sequence and determines the codon usage bias of the VP2 gene for the first time. Epidemiological surveillance is necessary to understand the importance and evolution of MiBoV1.

**Keywords** Mink bocavirus · VP2 gene · Codon usage · Phylogenetic analysis

## Introduction

Bocavirus (BoV), a species from the genus *Bocaparvovirus* and the family Parvoviridae, is an approximately 26-nm non-enveloped capsid virus with icosahedral symmetry. It has a

small linear single-stranded DNA genome comprising three open reading frames (ORFs) [1]. The first ORF encodes non-structural proteins associated with viral gene replication (NS1), whereas the second ORF encodes two capsid proteins (VP1 and VP2); the VP2 capsid protein is the major capsid protein and plays an important role in the determination of the antigenicity and host range of BoVs [2–5]. The third ORF encodes a highly phosphorylated nonstructural protein (NP1) [6]. BoVs are found in humans and various animals, including chimpanzees, gorillas, pigs, cows, bats, dogs, cats, rats, California sea lions, and pine martens [7–11], and they cause gastrointestinal and respiratory diseases in their hosts. When hosts are infected with BoV, disease symptoms tend to be severe in young animals and infants, whereas subclinical infection is common in adults [8, 10]. Canine bocavirus type 2 infection is associated with massive enteritis in a litter of dogs with atrophied and fused villi, severe crypt regeneration, severe bone marrow, and lymphoid atrophy [10]. Respiratory tract symptoms and diarrhea have been reported in weanling piglets infected with porcine bocavirus [12, 13]. BoVs are related to DNA damage, which may promote viral DNA replication and mediate cell death [14].

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Mink bocavirus 1 (MiBoV1), a novel species of BoV, was identified from the domestic minks in China in 2016 [15]. MiBoV1 was detected in healthy animals and animals suffering from diarrhea [15]; thus, the correlation between MiBoV1 and diarrhea requires further confirmation. Despite the availability of MiBoV sequences in clinical samples, the pathogenic role and prevalence of MiBoV1 in single or co-infection are unclear. Moreover, the genetic information, phylogenetic analysis, and codon usage pattern of this virus are limited.

In the present study, we collected 192 samples from the major mink industry province in China and detected the samples for the presence of MiBoV1 by PCR. Co-infection with MiBoV1 was also detected. Subsequently, the cloned VP2 sequences were compared between MiBoV and other known BoVs to investigate the variation of nucleotides and amino acids, and the genetic relationship of MiBoV1 to known BoV isolates was examined using phylogenetic analysis. In addition, the codon usage bias of the VP2 gene of MiBoV1 genome was measured by using bioinformatic methods.

## Materials and methods

### Sample collection

A total of 192 samples, including 26 sera from minks with septicemia, 39 diarrhea feces, and 127 tissues of dead animals, were collected from 23 farms in Liaoning, Jilin, Heilongjiang, Hebei, and Shandong provinces from May 2014 to September 2017. All samples originated from independent mink individuals aged 60–118 days, and the 127 tissues, including lungs, hearts, spleens, livers, kidneys, brains, etc., were screened from 127 dead animals. No duplicate samples were used (one tissue from one corpse). The details of the samples are listed in Supplementary Material Table S1.

### Laboratory investigation

Genomic DNA was extracted from feces and tissues [16]. Approximately 0.1 g of each specimen was added into 500  $\mu$ L of phosphate-buffered saline, homogenized, freeze thawed three times, and then centrifugated. Total genomic DNA was extracted from 200  $\mu$ L of homogenate supernatant by using genomic DNA Kit (Axygen A Corning Brand, Suzhou, China) in accordance with the manufacturer's protocol. Serum DNA was extracted by using the Serum/Plasma Circulating DNA Kit (TIANGEN BIOTECH CO., LTD.) in accordance with the manufacturer's instructions.

According to the published protocol, BoV was detected from 192 total viral DNA by using the reference method [15]. To detect co-infection, MiBoV1-positive samples were

also used to examine mink enteritis virus, mink circovirus (MiCV), and Aleutian mink disease virus (AMDV) by PCR, and the corresponding original samples were examined for canine distemper virus, canine adenovirus (CAV)-1, and CAV-2 using the colloidal gold immunochromatography strip.

### Amplification and sequencing of complete VP2 gene

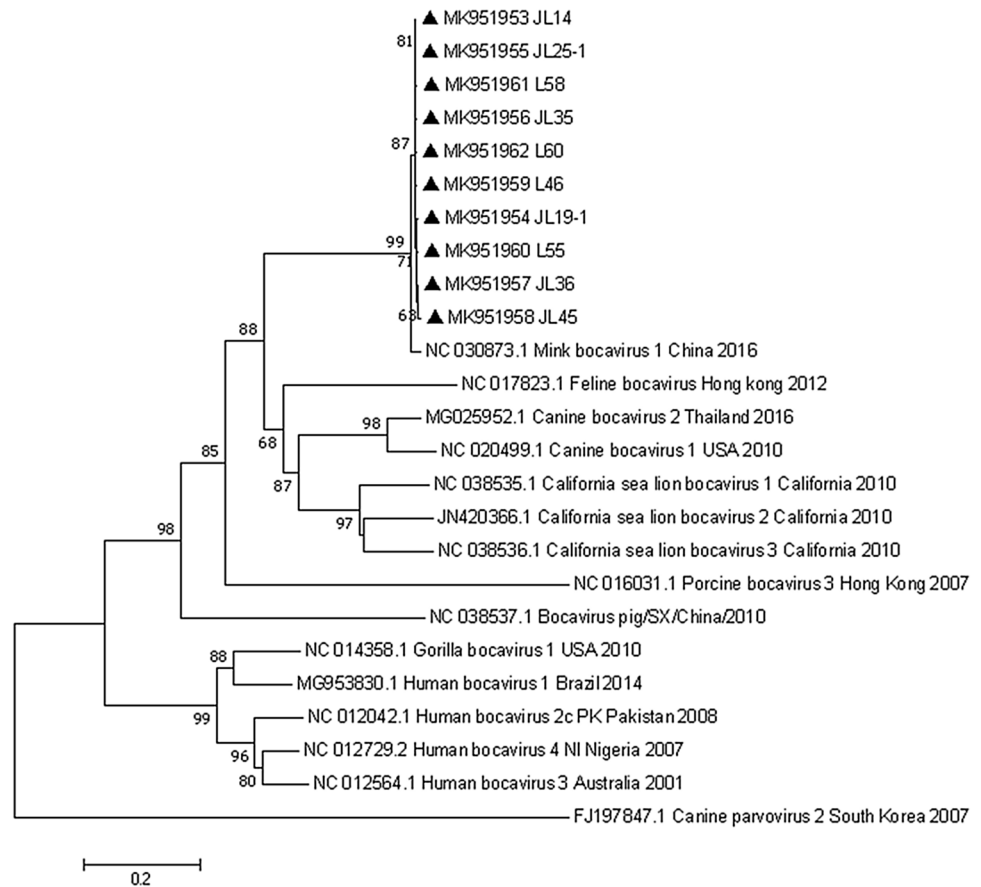
On the basis of the published VP2 gene sequence of the MiBoV strain (NC\_030873.1), the nested primers were designed to perform PCR screening. Primers Boca-F1 (5'-AAATCGGCGTTTAGACT-3') and Boca-R1 (5'-GGATAACAAGATATACATGAC-3') were used for the first round of PCR, and Boca-F2 (5'-CAAACCGTAAGGGAGGAG-3') and Boca-R2 (5'-GCTGGATATACATGACAGAAAT-3') were used for the second round. All primers were purchased from Comate Bioscience, Changchun, China. For the nested PCR, Ex Taq DNA polymerase (TaKaRa, Japan) was used. The first round of PCR was conducted at 95 °C for 5 min, followed by 35 cycles at 95 °C for 45 s, annealing at 51.3 °C for 2 min, elongation at 72 °C for 1 min, and final extension at 72 °C for 8 min. For the second round of nested PCR, the annealing temperature was 52 °C under identical cycling conditions.

PCR products were purified using a gel extraction kit (AXYGEN, Hangzhou, China) in accordance with the manufacturer's instructions. The purified DNA was cloned into a pMD18-T vector, the plasmids were introduced into *Escherichia coli* DH5 $\alpha$  by using a standard transformation technique, and the positive clones were sent to Comate Bioscience (Changchun, China) for sequencing. At least three independent plasmid clones were analyzed and confirmed.

### Genetic and phylogenetic analysis

The SeqMan program of DNASTar was used to edit and analyze the nucleotide sequences (DNASTar, Madison, WI, USA). The VP2 sequence of MiBoV1 obtained in this study had been deposited in GenBank (accession number: MK951953 to MK951962). The nucleotide and predicted amino acid sequences of complete VP2 sequences were multiple aligned between different species of BoV by Clustal W, MegAlign program of DNASTar (DNASTar, Madison, WI, USA). Subsequently, to improve our understanding of the genetic relationships between MiBoV1 and other BoVs, we constructed a phylogenetic and molecular evolution tree on the basis of the nucleotide sequences of all VP2 genes by MEGA 7.0 via the neighbor-joining method and Kimura 2-parameter model. The reliability of the phylogenetic tree obtained for the VP2 region was evaluated by running 1000 replicates in the bootstrap test (Fig. 1) [17, 18].

**Fig. 1** Phylogenetic analysis of the VP2 gene sequence of mink bocavirus and other bocavirus, using the neighbor-joining method, with 1000 bootstrap replicates (MEGA 7.0.14). Only bootstrap support values greater than 60% are shown. The bar indicates the genetic distance. Other sequences were obtained from GenBank; accession numbers of those sequences are included in the tree. The triangle represents 10 VP2 sequences in this study



## Codon usage pattern analysis

The basic nucleotide composition (A%, T%, C%, and G%), AT and GC contents, nucleotide at the third position of synonymous codons (A3s%, T3s%, C3s%, and G3s%), GC content on the third synonymous codon position (GC3s), relative synonymous codon usage (RSCU), and effective number of codons (ENC) were calculated for the VP2 gene sequence of each MiBoV1 strain by using CodonW version 1.4.2 [19]. The codon adaptation index (CAI) values for MiBoV1 were calculated using Neovison vison codon usage as a reference set in the CAI calculator <https://genomes.urv.es/CAIcal/> [20].

## Results

### Prevalence of MiBoV1

Among the 192 samples tested, 10 (5.2%) cases were positive for MiBoV1 by PCR. The positive minks aged 60–118 days were mostly characterized by vomiting, lethargy, anorexia, dehydration, diarrhea, and rough fur. Six of the 10 positive samples were from the sera of minks with

septicemia. The other four samples were from feces collected from minks with diarrhea or hemorrhagic diarrhea. The prevalence of MiBoV1 varied significantly among different provinces: Hebei (10.81%) and Jilin (13.04%). No positive reaction was detected in samples from Heilongjiang, Liaoning, and Shandong provinces. The detection of co-infection indicated that five (50%) samples were positive for MiCV, and AMDV was positive in two cases (20%). In one case, MiBoV1, AMDV, and MiCV were detected simultaneously. The information of positive samples, including collection date, age, location, accession number, and other enteric pathogens detected, is listed in Table 1.

### Genetic characterization

To compare the mutation between nucleotides and amino acids, we analyzed 11 VP2 sequences of MiBoV1, including the reference strain (NC\_030873.1), and 10 sequences with the Clustal W method using the MegAlign program (DNASTAR, Madison, WI, USA). As shown in Table 2, the nucleotide similarity varied from 96.7% to 99.9%. The lowest nucleotide sequence similarity of the MiBoV1 reference strain (NC\_030873.1) and strain JL45 was 96.7%, whereas the similarity of nucleotides between

**Table 1** The details about the positive samples from which the viruses were detected

Strain	Location	Sampling time	Age (days)	Sample type	Clinical symptoms	Accession number	Mink bocavirus PCR results		Other enteric pathogens detected
							1st PCR	Nested PCR	
L60	Hebei	JUL/2017	60	Feces	Diarrhea	MK951961	–	+	Mink circovirus
L58	Hebei	JUL/2017	67	Feces	Diarrhea	MK951960	–	+	None
L55	Hebei	JUL/2017	63	Feces	Diarrhea	MK951958	–	+	Mink circovirus, AMDV
JL45	Jilin	SEP/2017	112	Sera	Septicemia	MK951957	–	+	Mink circovirus
JL36	Jilin	SEP/2017	106	Sera	Septicemia	MK951962	–	+	None
JL35	Jilin	SEP/2017	118	Sera	Septicemia	MK951959	–	+	Mink circovirus
JL25-1	Jilin	AUG/2017	91	Sera	Septicemia	MK951953	–	+	None
JL19-1	Jilin	JUL/2017	62	Sera	Septicemia	MK951956	–	+	Mink circovirus
JL14	Jilin	JUL/2017	65	Sera	Septicemia	MK951955	–	+	None
L46	Hebei	SEP/2017	113	Feces	Diarrhea	MK951954	+	+	AMDV

strain JL25-1 and strain JL 14 was the highest (99.9%). The nucleotide sequence alignment was free of deletion or insertion. This finding suggested that the VP2 gene was highly conserved and had low viral diversity (Table 2).

The amino acid alignment revealed that some synonymous mutations were only seen in the MiBoV1 detected in this study compared with the MiBoV1 reference strain (NC\_030873.1). For instance, a G to A mutation at nt positions 42, 57, 630, 669, 1125, and 1416; A to G mutation at nt positions 213, 252, 399, 441, 447, 702, and 705; T to C mutation at nt positions 141, 384, 627, 1014, 1074, and 1284; C to T mutation at nt positions 573, 651, 660, 960, 1008, 1161, 1338, and 1414; A to T mutation at nt position 1335; and T to G mutation at nt position 1458 were found in 10 MiBoV1 isolates. Four of the isolates (L58, JL35, L60, and JL45) had unique nt changes: C84G, A96G, T1038 C, and G1599A, respectively.

Table 3 shows that compared with the MiBoV1 reference strain (NC\_030873.1), 11 nonsynonymous mutations, namely, A53G, G58T, G60A, T61A, A71G, G214T, G833A, T937G, G1024A, C1025G, and T1595G, led to nine amino acid mutations at positions 18 (Lys → Arg), 20 (Ala → Ser), 21 (Ser → Thr), 24 (Asn → Ser), 72 (Asp → Tyr), 278 (Ser → Asn), 313 (Ser → Ala), 342 (Ala → Arg), and 532 (Leu → Trp). These mutations were observed in all 10 MiBoV1 isolates described in this study. The strain n JL45 with the highest mutation rate possessed more amino acid changes at residues 177 (Pro → Arg), 548 (Leu → Pro), 565 (Glu → Asp), and 576 (Val → Ser). Strain L58 contained three nt changes, namely, C462A, T760C, and A1568G, which led to three amino acid mutations, that is, F154L, Y254H, and E523G. In addition, a unique A → G mutation was observed in VP2 gene at nt positions 530, 314, and 568 of strain JL45, JL35, and JL14 separately, which was different from other strains. The

high variability was located at nt positions 53–214 and 833–1595.

Compared with other BoV strains, the 10 VP2 exhibited 51.9–63.7% nucleotide sequence identity. In comparing strains L60, JL14, and JL 25–1 with Canine bocavirus 2 (CBoV2), the nucleotide identity was the highest (63.7%) and that of strain JL45 and gorilla bocavirus (GBoV1) was the lowest (51.9%). The VP2 sequence of porcine bocavirus (PBoVsx and PBoV3) and four types of human bocaviruses (HBoV1–4) were compared individually with those of MiBoV1, and 54.1–60.2% nucleotide identity and 40.0–46.9% amino acid similarity were found. By contrast, MiBoV1 was most closely related to Feline bocavirus (FBoV, 63.0–63.4% nucleotide identity, and 58.6–59.1% amino acid similarity), followed by the three types of California sea lion bocaviruses (CslBoV1–3, 62.2–63.1% nucleotide identity, and 59.2–62.8% amino acid similarity) (Table S3, Supplementary Material).

## Phylogenetic studies

To examine the phylogenetic relationships between MiBoV1, we constructed a phylogenetic tree on the basis of full-length VP2 gene sequences (Fig. 1). The phylogenetic analysis clearly demonstrated that MiBoV1 formed two clades. One clade included the reference sequence (NC\_030873.1), and the other clade contained 10 isolates in this study. The virus showed a trend of evolutionary divergence. All MiBoV1 strains were distributed in a group with CslBoV, CBoV, and FBoV, which was consistent with the results of nucleotide comparison (Table 2), indicating that these viruses may share a common ancestor. These results were verified using the maximum likelihood and maximum parsimony methods, which yielded the same tree topology. Yang et al. performed a phylogenetic

**Table 2** Comparison of nucleotide and amino acid sequence of the VP2 gene among bocavirus strains

Bocavirus	% Nucleotide sequence identity										
	Mink										
	NC_030873.1	L60	L58	L55	JL45	JL36	JL35	JL25-1	JL19-1	JL14	L46
% amino acid sequence identity											
NC_030873.1		97.2	97.1	96.9	96.7	97.2	97.0	97.2	97.2	97.2	97.0
L60	98.4		99.3	99.1	98.9	99.0	99.6	99.5	99.1	99.4	99.5
L58	97.9	99.3		99.3	98.6	99.2	99.3	99.8	99.4	99.7	99.2
L55	98.4	99.8	99.3		99.3	99.8	99.1	99.5	99.7	99.4	99.5
JL45	97.7	99.1	98.6	99.1		99.4	99.0	98.7	99.2	98.7	99.3
JL36	98.4	99.8	99.3	99.8	99.1		99.0	99.4	99.8	99.3	99.4
JL35	98.3	99.7	99.1	99.7	99.0	99.7		99.5	99.1	99.5	99.7
JL25-1	98.4	99.8	99.3	99.8	99.1	99.8	99.7		99.5	99.9	99.4
JL19-1	98.4	99.8	99.3	99.8	99.1	99.8	99.7	99.8		99.5	99.3
JL14	98.3	99.7	99.1	99.7	99.0	99.7	99.5	99.7	99.7		99.4
L46	98.4	99.8	99.3	99.8	99.1	99.8	99.7	99.8	99.8	99.7	
PBoVsx	46.6	46.9	46.9	46.9	46.9	46.9	46.9	46.9	46.9	46.9	46.9
FBoV	59.1	59.1	58.6	59.1	58.6	59.1	59.1	59.1	59.1	59.1	59.1
HBoV-1	45.9	46.1	45.6	46.1	45.8	46.1	45.9	46.1	46.1	46.1	46.1
CBoV1	59.6	60.1	59.6	60.1	59.4	60.1	59.9	60.1	60.1	60.1	60.1
GBoV1	44.8	45.2	44.6	45.2	44.8	45.2	45.0	45.2	45.2	45.2	45.2
CsIBoV1	62.7	62.8	62.3	62.8	62.1	62.8	62.6	62.8	62.8	62.8	62.8
Bocavirus	% nucleotide sequence identity										
	PBoVsx	FBoV	HBoV-1	CBoV1	GBoV1	CsIBoV1					
% amino acid sequence identity											
NC_030873.1	58.2	63.4	59.3	61.9	57.3	62.7					
L60	57.7	63.1	59.0	62.5	56.7	62.4					
L58	57.6	63.2	59.1	62.5	56.8	62.3					
L55	57.8	63.4	58.0	62.5	56.6	62.5					
JL45	57.5	63.1	58.9	62.2	51.9	62.3					
JL36	57.7	63.4	59.3	62.5	56.7	62.6					
JL35	57.8	63.0	57.9	62.4	56.7	62.4					
JL25-1	57.8	63.2	58.7	62.6	57.0	62.4					
JL19-1	57.5	63.4	59.2	62.6	56.8	62.4					
JL14	57.8	63.2	58.7	62.6	57.0	62.5					
L46	57.7	63.2	58.0	62.7	56.7	62.3					
PBoVsx		46.4	38.0	56.9	52.1	56.6					
FBoV	59.0		54.5	62.9	58.9	61.4					
HBoV-1	58.5	45.0		52.4	81.5	53.3					
CBoV1	46.0	60.3	43.0		53.0	68.5					
GBoV1	47.3	44.8	86.2	43.4		53.2					
CsIBoV1	37.8	61.9	45.0	67.1	45.2						

The GenBank accession numbers of different species bocaviruses are shown as follows. PBoV3, NC\_016031.1; PBoVsx, NC\_038537.1; FBoV, NC\_017823.1; HBoV-1, MG953830.1; HBoV-2c, NC\_012042.1; HBoV-3, NC\_012564.1; HBoV4-NI, NC\_012729.2; CBoV1, NC\_020499.1; CBoV2, MG025952.1; GBoV1, NC\_014358.1; CsIBoV1, NC\_038535.1; CsIBoV2, JN420366.1; CsIBoV3, NC\_038536.1

analysis on the basis of the NS1 amino acid sequences and found that MiBoV clustered with FBoV (JQ692585), CsIBoV1 (JN420361), and Canine bocavirus 1 (CBoV1, JN648103) and formed a distinct clade. This result is the

same with our phylogenetic tree based on the VP2 gene sequence.

**Table 3** Comparison of VP2 nucleotide and amino acid mutations of MiBoV1 isolates reported in this study

Isolate	Mutation sites: nucleotide (amino acid residue)																					
	53(18)	58(20)	60(20)	61(21)	71(24)	214(72)	314	462	530	568	760	833	937	1024	1025	1568	1595	1643	1695	1726	1727	
	(105)	(154)	(177)	(190)	(254)	(313)	(342)	(342)	(342)	(523)	(532)	(548)	(565)	(576)	(576)	(576)	(576)	(576)	(576)	(576)	(576)	
Mutation sequences: nucleotide (amino acid residue)																						
a → g	g → t	g → a	t → a	a → g	a → g	g → t	a → g	c → a	c → g	a → g	t → c	g → a	t → g	g → a	c → g	a → g	t → g	t → c	g → t	g → t	t → c	
(K → R)	(A → S)	(A → S)	(S → T)	(N → S)	(D → Y)	(Q → R)	(F → L)	(P → R)	(T → A)	(Y → A)	(Y → H)	(S → N)	(S → A)	(A → R)	(A → R)	(E → G)	(L → W)	(L → P)	(E → D)	(V → S)	(V → S)	
NC_030873.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
L60	g	t	a	a	g	t	-	-	-	-	-	a	a	a	g	-	g	-	-	-	-	
L58	g	t	a	a	g	t	-	-	-	c	-	g	g	g	g	g	g	-	-	-	-	
L55	g	t	a	a	g	t	-	-	-	-	-	g	g	g	g	g	g	-	-	-	-	
JL45	g	t	a	a	g	t	-	-	-	-	-	g	g	g	g	g	g	c	t	t	c	
JL36	g	t	a	a	g	t	-	-	-	-	-	g	g	g	g	g	g	-	-	-	-	
JL35	g	t	a	a	g	t	-	-	-	-	-	g	g	g	g	g	g	-	-	-	-	
JL25-1	g	t	a	a	g	t	-	-	-	-	-	g	g	g	g	g	g	-	-	-	-	
JL19-1	g	t	a	a	g	t	-	-	-	-	-	g	g	g	g	g	g	-	-	-	-	
JL14	g	t	a	a	g	t	-	-	-	-	-	g	g	g	g	g	g	-	-	-	-	
L46	g	t	a	a	g	t	-	-	-	-	-	g	g	g	g	g	g	-	-	-	-	

**Analysis of the codon usage pattern**

Basic nucleotide composition analysis was conducted for the VP2 gene of MiBoV1. The mean values of A% (32.21 ± 0.09) and G% (24.06 ± 0.07) were the highest, followed by C% (22.54 ± 0.26) and T% (21.28 ± 0.08), indicating a strong compositional bias in favor of A or G. The mean values of AT% and GC% were 53.49 ± 0.12 and 46.51 ± 0.12, respectively, whereas the mean value of GC3s% was 39 ± 0.30. According to the nucleotide composition, MiBoV1 VP2 genes were AG rich. Therefore, A and G seemed to be more common than T and C at the wobble position of VP2 gene sequences. However, further analysis of third position wobble nucleotides revealed a significantly higher abundance of A3s (44 ± 0.38%) and T3s (33 ± 0.30%) than C3s (26 ± 0.23%) and G3s (24 ± 0.26%).

Among the codons of the VP2 gene of 11 MiBoV1 strains (Table 4), codons with RSCU value > 2, including CCA, AGA, and UAA, were over-represented codons. This finding showed that proline, arginine, and the terminating codon of MiBoV1 VP2 gene over-preferred CCA, AGA, and UAA, respectively. The codons UUU (Phe), GUC (Val), CAU (His), CAA (Gln), AAU (Asn), AAA (Lys), GAA (Glu), UCA (Ser), CCA (Pro), ACG (Thr), GCA (Ala), and AGA (Arg) showed similar preferences in all MiBoV1 strains. The codons preferred by each MiBoV1 strain individually were as follows. The codons solely preferred in strains JL19-1, JL36, JL45, L46, and L55 and the reference sequence (NC\_030873.1) were UAU (Tyr), whereas strain L58 preferred UAC (Tyr). Codons solely preferred in MiBoV1 that originated from northern China were AUU (Ile), GAU (Asp), and UGC (Cys) for three of the amino acids, whereas the reference sequence (NC\_030873.1) preferred AUC (Ile) and UGU (Cys), showing no preference for synonymous codon usage in Asp. No bias for codons encoding Tyr was detected in strains JL14, JL25-1, JL35, and L60. The RSCU values and end nucleotide composition showed that in the VP2 coding region, A/U-ended codons were strongly preferred in MiBoV1. GC-ending codons were seldom found preferred. Further analysis revealed that most amino acids in MiBoV1 VP2 protein were terminated by A/U-ending codons. For example, six different codons encoded three amino acids, namely, arginine, serine, and leucine; however, in MiBoV1 VP2 protein, all preferred synonymous codons were terminated by A-ended codons.

The ENC value describes the degree to which codon usage deviates from random selection. It is inversely proportional to codon usage deviation and shows the bias from an equal use of all synonymous codons for a given amino acid. The ENC values of different MiBoV1 strains ranged from 51.64 to 52.77 with a mean value of 52.13 ± 0.4296, which indicated that high ENC values (> 40) [19] and minimal variation among different isolates suggested a moderate,

**Table 4** RSCU analysis of mink bocavirus VP2 gene

AA	Codon	JL14	JL19-1	JL25-1	JL35	JL36	JL45	L46	L55	L58	L60	NC_030873.1	AA	Codon	JL14	JL19-1	JL25-1	JL35	JL36	JL45	L46	L55	L58	L60	NC_030873.1	
Phe <sup>a</sup>	UUU	<b>1.50<sup>b</sup></b>	<b>1.50</b>	<b>1.50</b>	<b>1.50</b>	<b>1.50</b>	<b>1.50</b>	<b>1.50</b>	<b>1.50</b>	<b>1.58</b>	<b>1.50</b>	<b>1.50</b>	Ser	UCU	0.88	0.88	0.88	0.88	0.88	0.86	0.88	0.88	0.88	0.88	0.88	0.86
	UUC	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.42	0.50	0.50		UCC	0.35	0.35	0.35	0.35	0.35	0.69	0.53	0.35	0.35	0.35	0.35	0.34
	UUA	<b>1.57</b>	<b>1.57</b>	<b>1.57</b>	<b>1.30</b>	<b>1.57</b>	<b>1.36</b>	<b>1.30</b>	<b>1.57</b>	<b>1.75</b>	<b>1.75</b>	<b>1.30</b>	1.00	UCA	<b>1.59</b>	<b>1.41</b>	<b>1.59</b>	<b>1.41</b>	<b>1.41</b>	<b>1.20</b>	<b>1.41</b>	<b>1.59</b>	<b>1.59</b>	<b>1.41</b>	<b>1.20</b>	<b>1.20</b>
Leu	UUG	0.52	0.52	0.52	0.52	0.52	0.55	0.52	0.52	0.50	0.52	0.75		UCG	0.71	0.88	0.71	0.71	0.88	0.86	0.71	0.71	0.71	0.71	0.71	<b>1.20</b>
	CUU	0.52	0.52	0.52	0.52	0.52	0.55	0.52	0.52	0.50	0.52	0.25		AGU	1.24	1.24	1.24	1.24	1.24	1.24	1.24	1.24	1.24	1.24	1.24	<b>1.20</b>
	CUC	0.52	0.52	0.52	0.52	0.52	0.55	0.52	0.52	0.50	0.52	0.75		AGC	1.24	1.24	1.24	1.24	1.24	1.24	1.24	1.24	1.24	1.24	1.24	<b>1.20</b>
Ile	CUA	<b>1.57</b>	<b>1.57</b>	<b>1.57</b>	<b>1.83</b>	<b>1.57</b>	<b>1.91</b>	<b>1.83</b>	<b>1.57</b>	1.50	<b>1.83</b>	<b>1.75</b>	Pro	CCU	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59
	CUG	1.30	1.3	1.30	1.30	1.30	1.09	1.30	1.30	1.25	1.30	1.50		CCC	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
	AU	<b>1.38</b>	<b>1.5</b>	<b>1.38</b>	<b>1.38</b>	<b>1.50</b>	<b>1.50</b>	<b>1.50</b>	<b>1.50</b>	<b>1.50</b>	<b>1.38</b>	<b>1.38</b>	1.27	CCA	<b>2.73</b>	<b>2.73</b>	<b>2.73</b>	<b>2.73</b>	<b>2.73</b>	<b>2.63</b>	<b>2.73</b>	<b>2.73</b>	<b>2.73</b>	<b>2.73</b>	<b>2.63</b>	<b>2.73</b>
Val	AUC	1.27	1.15	1.27	1.15	1.15	1.15	1.15	1.15	1.27	1.27	<b>1.38</b>		CCG	0.49	0.49	0.49	0.49	0.49	0.59	0.49	0.49	0.49	0.49	0.49	0.49
	AUA	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	Thr	ACU	0.88	0.87	0.87	0.87	0.93	0.93	0.93	0.93	0.87	0.87	0.87	0.95
	GUU	0.83	0.69	0.83	0.83	0.69	0.71	0.83	0.69	0.83	0.83	0.83		ACC	0.54	0.53	0.53	0.53	0.53	0.53	0.53	0.53	0.53	0.53	0.53	0.54
Tyr	GUC	<b>1.66</b>	<b>1.79</b>	<b>1.66</b>	<b>1.52</b>	<b>1.79</b>	<b>1.57</b>	<b>1.52</b>	<b>1.79</b>	<b>1.66</b>	<b>1.52</b>	<b>1.66</b>		ACA	1.08	1.13	1.13	1.13	1.13	1.13	1.13	1.13	1.13	1.13	1.13	1.08
	GUA	0.97	0.97	0.97	1.10	0.97	1.14	1.10	0.97	0.97	1.10	0.97		ACG	<b>1.49</b>	<b>1.47</b>	<b>1.47</b>	<b>1.47</b>	<b>1.47</b>	<b>1.40</b>	<b>1.40</b>	<b>1.40</b>	<b>1.47</b>	<b>1.47</b>	<b>1.47</b>	<b>1.42</b>
	GUG	0.55	0.55	0.55	0.55	0.55	0.57	0.55	0.55	0.55	0.55	0.55	Ala	GCU	1.19	1.22	1.22	1.22	1.22	1.22	1.22	1.22	1.22	1.22	1.22	1.30
His	UAU	1.00	<b>1.06</b>	1.00	1.00	<b>1.06</b>	<b>1.06</b>	<b>1.06</b>	<b>1.06</b>	0.94	<b>1.03</b>	1.00	1.10	GCC	0.35	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.76
	UAC	1.00	0.94	1.00	1.00	0.94	0.94	0.94	0.94	1.03	1.00	0.90		GCA	<b>1.51</b>	<b>1.44</b>	<b>1.44</b>	<b>1.44</b>	<b>1.56</b>	<b>1.56</b>	<b>1.44</b>	<b>1.44</b>	<b>1.56</b>	<b>1.44</b>	<b>1.33</b>	<b>1.41</b>
	CAU	<b>1.07</b>	<b>1.20</b>	<b>1.07</b>	<b>1.07</b>	<b>1.20</b>	<b>1.20</b>	<b>1.07</b>	<b>1.20</b>	<b>1.13</b>	<b>1.07</b>	<b>1.07</b>		GCG	0.54	0.56	0.56	0.56	0.44	0.44	0.44	0.56	0.44	0.56	0.56	0.54
Gln	CAC	0.93	0.80	1.00	0.93	0.80	0.80	0.93	0.80	0.88	0.93	0.93	Cys	UGU	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80
	CAA	<b>1.08</b>	<b>1.08</b>	<b>1.08</b>	<b>1.04</b>	<b>1.08</b>	<b>1.08</b>	<b>1.08</b>	<b>1.08</b>	<b>1.08</b>	<b>1.08</b>	<b>1.08</b>		UGC	<b>1.20</b>	<b>1.20</b>	<b>1.20</b>	<b>1.20</b>	<b>1.20</b>	<b>1.20</b>	<b>1.20</b>	<b>1.20</b>	<b>1.20</b>	<b>1.20</b>	<b>1.20</b>	0.80
	CAG	0.92	0.92	0.92	0.96	0.92	0.92	0.92	0.92	0.92	0.92	0.92	Arg	CGU	0.58	0.58	0.58	0.58	0.58	0.56	0.58	0.58	0.58	0.58	0.58	0.62
Asn	AAU	<b>1.19</b>	<b>1.24</b>	<b>1.19</b>	<b>1.19</b>	<b>1.19</b>	<b>1.19</b>	<b>1.14</b>	<b>1.19</b>	<b>1.19</b>	<b>1.19</b>	<b>1.19</b>		CGC	0.58	0.58	0.58	0.58	0.58	0.56	0.58	0.58	0.58	0.58	0.58	0.62
	AAC	0.81	0.76	0.81	0.81	0.81	0.81	0.86	0.81	0.81	0.81	0.81		CGA	0.58	0.58	0.58	0.58	0.94	0.94	0.58	0.58	0.58	0.58	0.58	0.62
	AAA	<b>1.18</b>	<b>1.18</b>	<b>1.18</b>	<b>1.06</b>	<b>1.18</b>	<b>1.06</b>	<b>1.06</b>	<b>1.18</b>	<b>1.18</b>	<b>1.18</b>	<b>1.33</b>		CGG	0.19	0.19	0.19	0.19	0.19	0.00	0.19	0.19	0.19	0.19	0.19	0.21
Lys	AAG	0.82	0.82	0.82	0.94	0.82	0.94	0.94	0.82	0.82	0.94	0.67		AGA	<b>3.29</b>	<b>3.29</b>	<b>3.29</b>	<b>3.19</b>	<b>3.29</b>	<b>3.19</b>	<b>3.29</b>	<b>3.29</b>	<b>3.29</b>	<b>3.29</b>	<b>3.29</b>	<b>3.31</b>
	GAU	<b>1.03</b>	<b>1.03</b>	<b>1.03</b>	<b>1.03</b>	<b>1.03</b>	<b>1.07</b>	<b>1.03</b>	<b>1.03</b>	<b>1.03</b>	<b>1.03</b>	1.00		AGG	0.77	0.77	0.77	0.75	0.75	0.75	0.77	0.77	0.77	0.77	0.62	
	GAC	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	1.00														
Glu	GAA	<b>1.41</b>	<b>1.41</b>	<b>1.41</b>	<b>1.41</b>	<b>1.41</b>	<b>1.46</b>	<b>1.41</b>	<b>1.41</b>	<b>1.41</b>	<b>1.38</b>	<b>1.41</b>														
	GAG	0.59	0.59	0.59	0.59	0.59	0.54	0.59	0.59	0.62	0.59	0.52														

AA amino acid

Nondegenerate amino acids (Met, Trp) and stop codons are excluded

The boldface are the most preferred codons

but highly conserved codon usage bias in the VP2 gene of MiBoV1.

To compare the codon usage preference with respect to its host, we calculated the CAI values for MiBoV1 VP2 genes by using mink (*Neovison vison*) codon usage as the reference. The closer the CAI to 1, the higher the gene expression level. The CAI varied from 0.626 to 0.63, with an average of  $0.63 \pm 0.0016$ . These results indicated that the VP2 genes were slightly expressive in the host and had lower adaptability to minks.

## Discussion

Despite scientific evidence proving that BoV exists in minks [15, 21], the prevalence, pathogenic role, and genetic characterizations of this virus are limited. In the present study, the detection results improve our understanding of the geographical distribution and tissue distribution of MiBoV1. The positive rates were 10.81% and 13.04% in the samples collected from Hebei and Jilin provinces, respectively. However, no positive samples were detected in the samples collected from Shandong, Liaoning, and Heilongjiang provinces. These results showed that the overall positive rate was low, and differences in the positive rate among different regions were found. In addition, MiBoV1 was detected for the first time in serum samples, not only in feces.

To further examine the tissue distribution of virus, we also tested other tissues corresponding to the positive serum samples. The tissue samples we tested included the liver, lung, spleen, heart, duodenum, jejunum, ileum, colon, brain, kidney, skeletal muscle, bone marrow, and mesenteric lymph nodes; however, BoV was not found in these tissues. This result suggested that viruses could enter the bloodstream through some mechanism to cause viremia, but why they cannot enter other tissues needs further study. Another finding showed that co-infection of MiBoV1 with Aleutian virus and circovirus existed in 60% of all MiBoV1-positive samples. These findings indicate that the virus may have only limited pathogenicity. However, considering that these samples show septicemia in clinics, the relationship between the virus and the occurrence of septicemia should be studied.

Pairwise comparative genetic variation analysis of MiBoV1 showed that the nucleotide sequence identities of VP2 between different MiBoV1 strains were 96.7–99.9%, the amino acid sequence similarity ranged from 97.7% to 99.8%, and the mutation rate was consistent with that of other parvoviruses [22–24]. The MiBoV1 sequence in this study had 96.7–97.2% identity with the reference strain (NC\_030873.1) identified in China in 2016 [21] and had lower nucleotide (51.9–63.7%) and amino acid (40.0–62.8%) similarity compared with other BoVs of different species (Table S3, Supplementary Material).

Yang et al. performed a phylogenetic analysis on the basis of the NS1 amino acid sequences and found that MiBoV clustered with FBoV (JQ692585), CslBoV1 (JN420361), and CBoV1 (JN648103) formed a distinct clade. This finding was consistent with the results of VP2 gene sequence analysis in this study.

The VP2 capsid protein of BoV plays an important role in viral pathogenicity and host range of BoVs and includes major antigenic determinants. Thus, it can be used as an antigen for detection methods and in designing new generation vaccines. Through amino acid comparison analysis, we found that MiBoV1 had two evident hypervariable regions. One located at amino acid position 278–532 was consistent with parvoviruses [18, 22, 23]. The other hypervariable region at amino acid position 18–72 was distinctive. In addition, some strains had unique amino acid point mutations. The strain JL45 had amino acid changes at residues 177 (Pro → Arg), 548 (Leu → Pro), 565 (Glu → Asp), and 576 (Val → Ser). Strain L58 exhibited three nt changes C462A, T760C, and A1568G, which resulted in three nucleotide mutations, namely, F154L, Y254H, and E523G. A unique A-G mutation at position 314 of JL35, position 530 of strain JL45, position 1568 of strain L58, and position 568 of JL14 was different from other strains. At present, the viral diversity was low, and the VP2 sequence of MiBoV1 was highly conservative. Therefore, the conservation of VP2 gene indicates that the MiBoV1 has maintained a certain genetic stability, which will lay a foundation for vaccine research.

The codon usage pattern in viruses can offer insights into improving our understanding of the evolution and pathogenesis of some viruses. The codon usage bias affects the structure and function of protein. In our study, we analyzed and compared the codon usage data of VP2 in MiBoV1. The results of codon usage analysis showed that MiBoV1 is AG rich, and most of the preferred codons terminated with either an A or U nucleotide. The unequal usage of A3s/T3s and G3s/C3s nucleotides in AG-rich VP2 genes in the present study indicated that the compositional patterns of the MiBoV1 VP2 genes were more complex than the commonly observed GT- and/or AG-rich compositions of most virus genes (Table S2, Supplementary Material). Our analysis also showed that although some differences exist in codon usage deviations among different isolates of MiBoV1, the average ENC, CAI, and GC3s values showed that codon usage bias in VP2 genes in MiBoV1 was less. The results were identical to the previously reported data on HBoV and other parvoviruses [25, 26]. Weak codon bias may be an adaptive strategy adopted by a great quantity of RNA and DNA viruses to not compete for limited tRNA resources and to maintain the efficient replication of viruses [27, 28].

To study the usage pattern of synonymous codons and the preferred degree of A/T termination codons, we calculated the RSCU of each codon in the VP2 gene of MiBoV1



(Table 4). Studying the use of synonymous codons in viruses can reveal the molecular evolution information of individual genes, which may help us understand the regulation of viral gene expression [27, 29]. In most studies, mutation bias is the major factor causing codon usage changes in genes with high A + T or G + C content [30–33]. A more comprehensive analysis is needed to reveal the true extent of codon usage bias variation within and among MiBoV1 isolates and what other factors are responsible, as well as the effects of factors such as cell tropism, principal host species, method of transmission, and viral genetic structure [26]. This information will enable us to more accurately determine the relative importance of mutation pressure and natural selection in determining the base composition and codon usage of these pathogens.

The relationship between MiBoV1 and diseases needs to be established. In this study, we only detected common viral pathogens, and one limitation is that no bacterial or other pathogens were tested in our experiment. Therefore, further studies are needed to identify the economic importance and pathogenicity of this emerging animal virus.

## Conclusions

To our knowledge, this is the first study to find and characterize MiBoV1 from serum. The results indicated that MiBoV1 had a low prevalence in the farm in Jilin and Hebei provinces. In addition, our study found divergence over the evolution of MiBoV1, and nucleotide and amino acid sequence analysis of MiBoV showed overall stability and individual variation. The codon usage bias of the VP2 gene in MiBoV1 was determined by bioinformatics method, which showed that the MiBoV1 VP2 gene was more likely to use codons that ended with A or U, and no obvious codon usage bias was found. The RSCU values and end nucleotide composition showed that in the VP2 coding region, A/U-ended codons were strongly preferred in MiBoV1. However, the pathogenic role of MiBoV1 in single or polymicrobial infections must be determined, and the economic importance of this virus is yet to be elucidated. Additional detailed studies must be conducted.

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**Author contributions** The submitting author confirms that all individual co-authors have met the criteria of authorship. YL, YY, TS, and LN contributed to sample collection and conducted experiments.

WX drafted the manuscript and analyzed data. JG critically revised the manuscript and gave final approval.

## Compliance with ethical standards

**Conflict of interest** The authors declare no potential conflicts of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors. Samples were collected from animals only for laboratory analyses, avoiding unnecessary pain and suffering of the animals. The mink farmers gave their written consent for sample collection.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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