Supplementary Materials for

The evolution of the coronavirus spike protein in the full-length genome and defective viral genome under diverse selection pressures

Ching-Hung Lin[1a], Hon-Man-Herman Tam[2a], Cheng-Yao Yang[1], Feng-Cheng Hsieh[1], Jiun-Long Wang[3,4,5], Chun-Chun Yang[1], Hsuan-Wei Hsu[1], Hao-Ping Liu[2*] and Hung-Yi Wu[1*]

a: Equal contributors

1 Graduate Institute of Veterinary Pathobiology, College of Veterinary Medicine, National Chung Hsing University, Taichung 40227, Taiwan

2 Department of Veterinary Medicine, College of Veterinary Medicine, National Chung Hsing University, Taichung 40227, Taiwan

3 Division of Chest Medicine, Department of Internal Medicine, Taichung Veterans General Hospital, Taichung 40705, Taiwan

4 Department of Post-Baccalaureate Medicine, College of Medicine, National Chung Hsing University, Taichung 40227, Taiwan

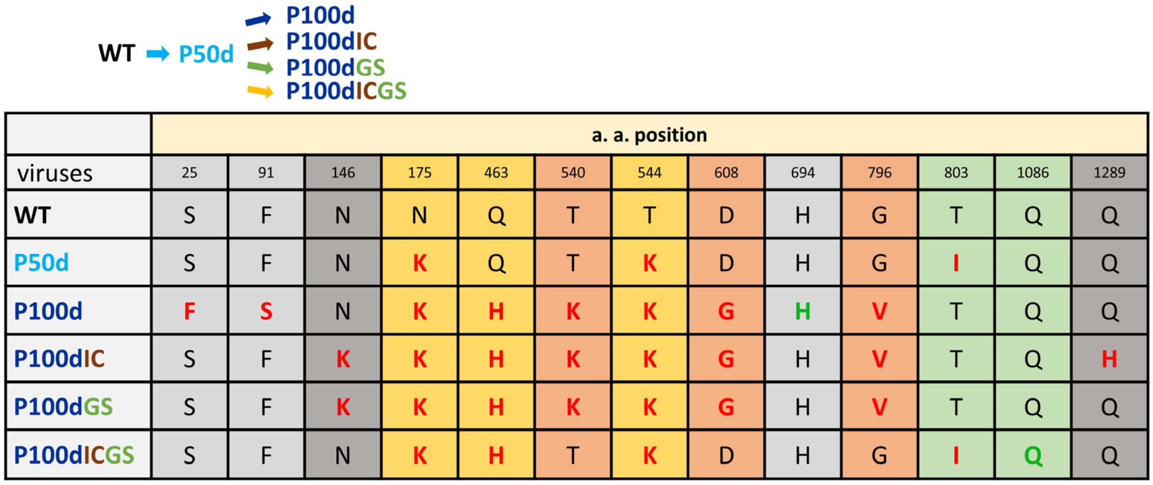
5 Department of Life Sciences, National Chung Hsing University, Taichung 40227, Taiwan

*Corresponding Author

Corresponding Footnote: Graduate Institute of Veterinary Pathobiology and Department of Veterinary Medicine, College of Veterinary Medicine, National Chung Hsing University, Taichung 40227, Taiwan

Telephone: 886-4-22840369; Fax: 886-4-22862073

Email: hwu2@dragon.nchu.edu.tw; hpliu@dragon.nchu.edu.tw



	a. a. position												
viruses	25	91	146	175	463	540	544	608	694	796	803	1086	1289
WT	S	F	N	N	Q	T	T	D	H	G	T	Q	Q
P50d	S	F	N	**K**	Q	T	**K**	D	H	G	**I**	Q	Q
P100d	**F**	**S**	N	**K**	**H**	**K**	**K**	**G**	**H**	**V**	T	Q	Q
P100dIC	S	F	**K**	**K**	**H**	**K**	**K**	**G**	H	**V**	T	Q	**H**
P100dGS	S	F	**K**	**K**	**H**	**K**	**K**	**G**	H	**V**	T	Q	Q
P100dICGS	S	F	N	**K**	**H**	T	**K**	D	H	G	**I**	**Q**	Q

Fig. S1. Amino acid sequence analysis of the S protein gene in the BCoV full-length genome under diverse selection pressure(s). The mutated amino acids are indicated in red. WT, wild-type BCoV without selection pressure; P50d, persistence at 50 d; P100d, persistence at 100 d; P100dIC, persistence and innate immunity treatment at 100 d; P100dGS, persistence and antiviral treatment at 100 d; P100dICGS, persistence, innate immunity and antiviral treatment at 100 d. a.a., amino acid.

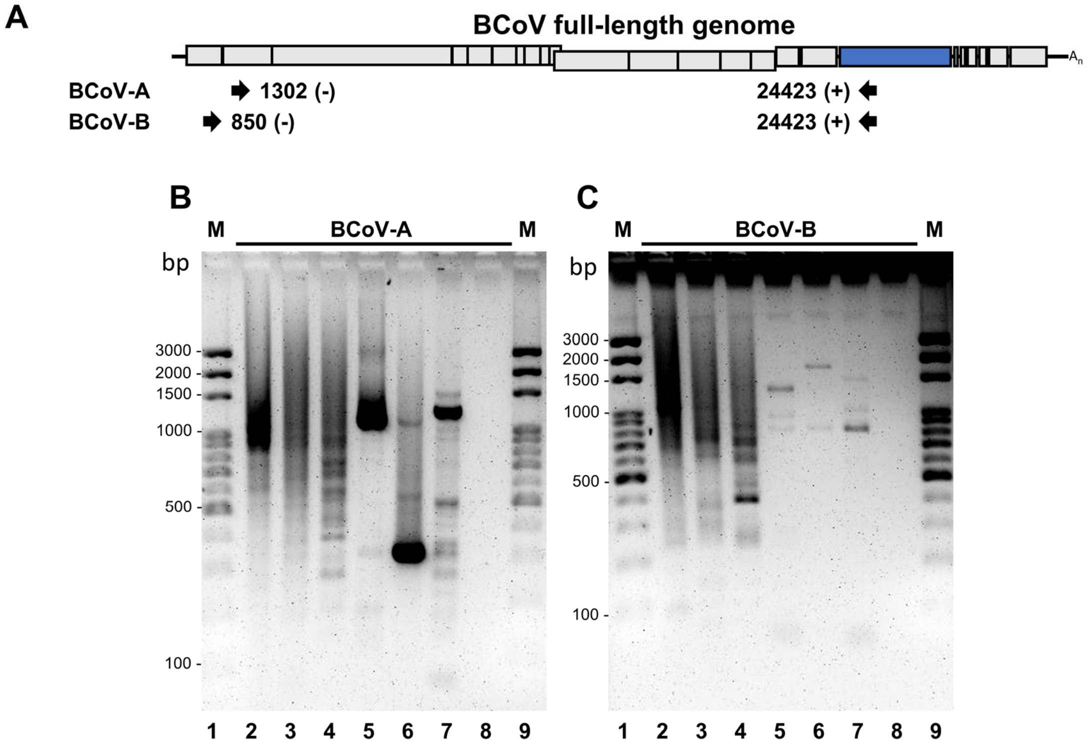


Fig. S2. The species and amounts of DVGs are altered under different selection pressures. **(A)** Diagram depicting the primer sets used for determining the synthesis of BCoV DVGs in Figs. (B)-(C)**.** **(B)-(C)** Detection of BCoV DVG synthesis under different selection pressures by RT-PCR. Lanes 1 and 9: DNA size marker; Lane 2: wild-type BCoV without selection pressure; Lane 3: P50d, persistence at 50 d; Lane 4: P100d, persistence at 100 d; Lane 5: P100dIC, persistence and innate immunity treatment at 100 d; Lane 6: P100dGS, persistence and antiviral treatment at 100 d; Lane 7: P100dICGS, persistence, innate immunity and antiviral treatment at 100 d: Lane 8: mock infection. bp, base-pair; M, DNA size marker.

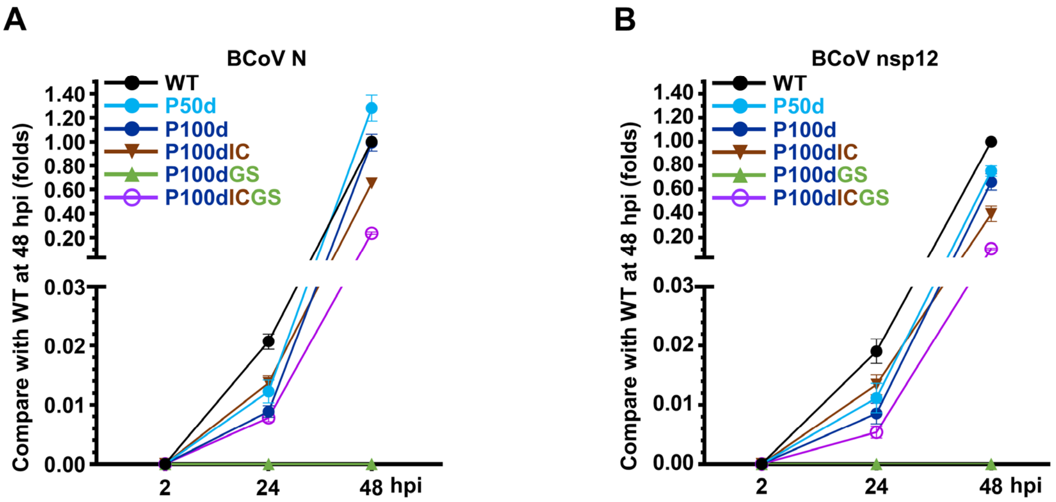
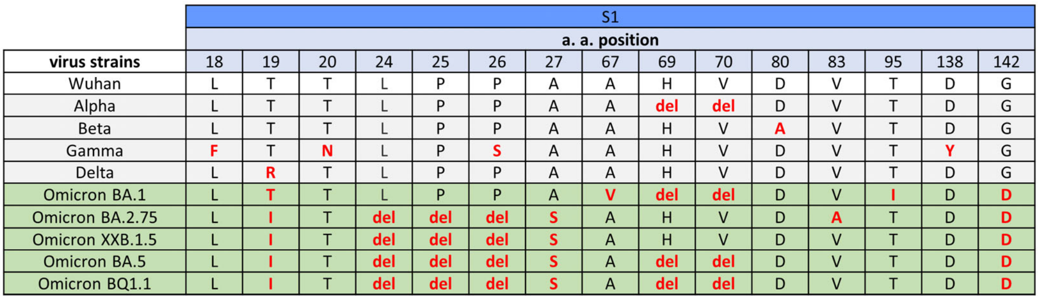
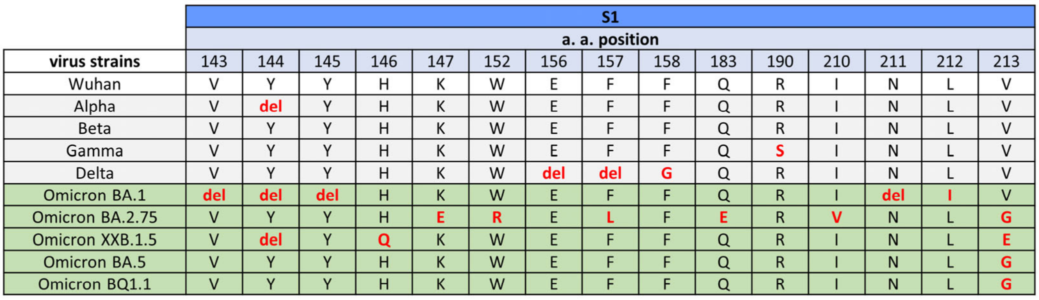


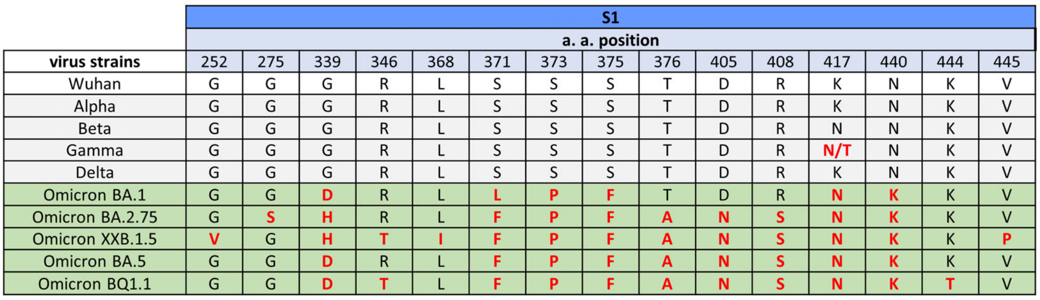
Fig. S3. The relative replication efficiency between the WT and variants, including P50d, P100d, P100dIC, P100dGS and P100dICGS under regular infection conditions, as quantitated by RT–qPCR for N protein (A) and nsp12 genes (B). hpi, hours postinfection. The values represent the means ± SDs from three individual experiments. ***$p < 0.001$.



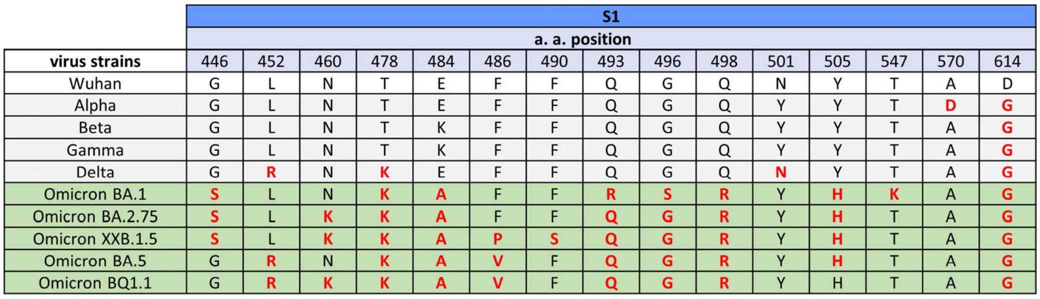
	S1														
	a. a. position														
virus strains	18	19	20	24	25	26	27	67	69	70	80	83	95	138	142
Wuhan	L	T	T	L	P	P	A	A	H	V	D	V	T	D	G
Alpha	L	T	T	L	P	P	A	A	del	del	D	V	T	D	G
Beta	L	T	T	L	P	P	A	A	H	V	A	V	T	D	G
Gamma	F	T	N	L	P	S	A	A	H	V	D	V	T	Y	G
Delta	L	R	T	L	P	P	A	A	H	V	D	V	T	D	G
Omicron BA.1	L	T	T	L	P	P	A	V	del	del	D	V	I	D	D
Omicron BA.2.75	L	I	T	del	del	del	S	A	H	V	D	A	T	D	D
Omicron XXB.1.5	L	I	T	del	del	del	S	A	H	V	D	V	T	D	D
Omicron BA.5	L	I	T	del	del	del	S	A	del	del	D	V	T	D	D
Omicron BQ1.1	L	I	T	del	del	del	S	A	del	del	D	V	T	D	D



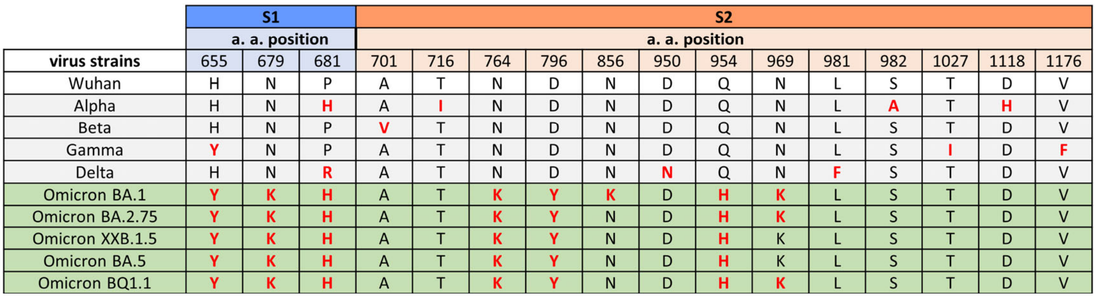
	S1														
	a. a. position														
virus strains	143	144	145	146	147	152	156	157	158	183	190	210	211	212	213
Wuhan	V	Y	Y	H	K	W	E	F	F	Q	R	I	N	L	V
Alpha	V	del	Y	H	K	W	E	F	F	Q	R	I	N	L	V
Beta	V	Y	Y	H	K	W	E	F	F	Q	R	I	N	L	V
Gamma	V	Y	Y	H	K	W	E	F	F	Q	S	I	N	L	V
Delta	V	Y	Y	H	K	W	del	del	G	Q	R	I	N	L	V
Omicron BA.1	del	del	del	H	K	W	E	F	F	Q	R	I	del	I	V
Omicron BA.2.75	V	Y	Y	H	E	R	E	L	F	E	R	V	N	L	G
Omicron XXB.1.5	V	del	Y	Q	K	W	E	F	F	Q	R	I	N	L	E
Omicron BA.5	V	Y	Y	H	K	W	E	F	F	Q	R	I	N	L	G
Omicron BQ1.1	V	Y	Y	H	K	W	E	F	F	Q	R	I	N	L	G



	S1														
	a. a. position														
virus strains	252	275	339	346	368	371	373	375	376	405	408	417	440	444	445
Wuhan	G	G	G	R	L	S	S	S	T	D	R	K	N	K	V
Alpha	G	G	G	R	L	S	S	S	T	D	R	K	N	K	V
Beta	G	G	G	R	L	S	S	S	T	D	R	N	N	K	V
Gamma	G	G	G	R	L	S	S	S	T	D	R	N/T	N	K	V
Delta	G	G	G	R	L	S	S	S	T	D	R	K	N	K	V
Omicron BA.1	G	G	D	R	L	L	P	F	T	D	R	N	K	K	V
Omicron BA.2.75	G	S	H	R	L	F	P	F	A	N	S	N	K	K	V
Omicron XXB.1.5	V	G	H	T	I	F	P	F	A	N	S	N	K	K	P
Omicron BA.5	G	G	D	R	L	F	P	F	A	N	S	N	K	K	V
Omicron BQ1.1	G	G	D	T	L	F	P	F	A	N	S	N	K	T	V



	S1														
	a. a. position														
virus strains	446	452	460	478	484	486	490	493	496	498	501	505	547	570	614
Wuhan	G	L	N	T	E	F	F	Q	G	Q	N	Y	T	A	D
Alpha	G	L	N	T	E	F	F	Q	G	Q	Y	Y	T	D	G
Beta	G	L	N	T	K	F	F	Q	G	Q	Y	Y	T	A	G
Gamma	G	L	N	T	K	F	F	Q	G	Q	Y	Y	T	A	G
Delta	G	R	N	K	E	F	F	Q	G	Q	N	Y	T	A	G
Omicron BA.1	S	L	N	K	A	F	F	R	S	R	Y	H	K	A	G
Omicron BA.2.75	S	L	K	K	A	F	F	Q	G	R	Y	H	T	A	G
Omicron XXB.1.5	S	L	K	K	A	P	S	Q	G	R	Y	H	T	A	G
Omicron BA.5	G	R	N	K	A	V	F	Q	G	R	Y	H	T	A	G
Omicron BQ1.1	G	R	K	K	A	V	F	Q	G	R	Y	H	T	A	G



	S1			S2												
	a. a. position			a. a. position												
virus strains	655	679	681	701	716	764	796	856	950	954	969	981	982	1027	1118	1176
Wuhan	H	N	P	A	T	N	D	N	D	Q	N	L	S	T	D	V
Alpha	H	N	H	A	I	N	D	N	D	Q	N	L	A	T	H	V
Beta	H	N	P	V	T	N	D	N	D	Q	N	L	S	T	D	V
Gamma	Y	N	P	A	T	N	D	N	D	Q	N	L	S	I	D	F
Delta	H	N	R	A	T	N	D	N	N	Q	N	F	S	T	D	V
Omicron BA.1	Y	K	H	A	T	K	Y	K	D	H	K	L	S	T	D	V
Omicron BA.2.75	Y	K	H	A	T	K	Y	N	D	H	K	L	S	T	D	V
Omicron XXB.1.5	Y	K	H	A	T	K	Y	N	D	H	K	L	S	T	D	V
Omicron BA.5	Y	K	H	A	T	K	Y	N	D	H	K	L	S	T	D	V
Omicron BQ1.1	Y	K	H	A	T	K	Y	N	D	H	K	L	S	T	D	V

Fig. S4. Sequencing analysis of the S protein in SARS-CoV-2 full-length genome during pandemics. SARS-CoV-2 reference genomes: Wuhan, GenBank: NC_045512.2; Alpha B.1.1.7, GenBank: QWE88920.1; Beta B.1.351, GenBank: QRN78347.1; Gamma P.1, GenBank: QRX39425.1; Delta B.1.617.2, GenBank: QUD52764.1; Omicron BA.1, GenBank: UFO69279.1; Omicron BA.2.75, GenBank: UTM82166.1; Omicron XBB.1.5, GenBank: UZG29433.1; Omicron BA.5, GenBank: UOZ45804.1; Omicron BQ.1.1, GenBank: UWM38596.1). Emergence: Alpha B.1.1.7 (UK, Sep 2020); Beta, B.1.351 (South Africa, Aug 2020); Gamma P.1 (Brazil, Jul 2020); Delta B.1.617.2 (India, Dec 2020); Omicron BA.1 (South Africa, Dec 2021); Omicron BA.2.75 (India, spring 2022); Omicron XBB.1.5 (USA, Nov 2022); Omicron BA.5 (South Africa, Jan 2022); BQ.1.1 (Nigeria, Jul 2022).

Table S1. Oligonucleotides used in the study

Oligonucleotides[a]	Polarity[b]	Sequence (5’ to 3’)
BCV 809(+)	—	cgtttatgatctttattgtagg
BCV 1050(+)	—	gctccaaataggaaataatgcc
BCV 1365(+)	—	cttgttcgcagtcaatataaccaaactgc
BCV 1637(+)	—	cgtgctggactccaatacac
BCV 1992(+)	—	caaataatatgcgcgcggaacc
BCV 3114(+)	—	gtcatcatcttcaggagctg
BCV 21000(+)	—	gtactcccctatgttcttag
BCV 21637(+)	—	gcaagactaatgtgcggcgctg
BCV 24146(+)	—	ccaactgtgatttacatgc
BCV 24239(+)	—	gtaatcagcattcacatcatatg
BCV 24286(+)	—	gcataaaaagtaccacc
BCV 24423(+)	—	ccaatattctaaagtcatagc
BCV 24556(+)	—	cgtttaattcataaacacc
BCV 24648(+)	—	gcaccgacttatcattaagc
BCV 24778(+)	—	cacataccatatatcttagc
BCV 25229(+)	—	cattgggcagcattatggcaag
BCV 25753(+)	—	cgtaattgcatttaatattccg
BCV 26047(+)	—	cctatagtaaactctgaagg
BCV 26298(+)	—	cgtctacattgaaattaacg
BCV 26548(+)	—	ggtggcagccaaagtgtatcc
BCV 26908(+)	—	gtctgtctatctgagcttgcgcttc
BCV 27038(+)	—	ccattaccacaaaaatttatcc
BCV 27853(+)	—	gttatataagacacagtacccac
BCV 27979(+)	—	cagtggtgctagtgtcaatacc
BCV END 2(+)	—	gtgattcttccaattggccataattaacttc

a The positive symbols in the oligonucleotide names indicate the polarities of the nucleic acids to which the oligonucleotides anneal.

b Polarity of the oligonucleotide relative to the positive-strand viral genome.

Table S1. Oligonucleotides used in the study (*Continued*)

Oligonucleotides[a]	Polarity[b]	Sequence (5’ to 3’)
BCV 20(-)	+	gattgtgagcgatttgcgtgc
BCV 7-32(-)	+	gagcgatttgcgtgcgtgcatcccgcttcact
BCV 21-51(-)	+	cgtgcatcccgcttcactgatctcttgttag
BCV 39(-)	+	gatctcttgttagatctttttataatctaaacttta
BCV 81(-)	+	catccactccctgtattctatgcttgtggg
BCV 126(-)	+	gtggtgtctatattcatttc
BCV 157(-)	+	gctttcagccagggacgtgttgtatcctaggc
BCV 426(-)	+	gcagtctagcctaatacgtg
BCV 443 (-)	+	gtgaaattgttatgaatacacg
BCV 450(-)	+	tgttatgaatacacgtccatatg
BCV 500(-)	+	tgcagtcccgcgaagcagttttgg
BCV 550(-)	+	gaggcatgctatgtgagaggttgt
BCV 577(-)	+	cctaatggatggaccatgggtttg
BCV 590(-)	+	ccatgggtttgtttcggcgtagaa
BCV 628(-)	+	ggtcgttgcgctgttaacaagc
BCV 650(-)	+	atgtggcctatcagctatatatga
BCV 679(-)	+	cctgcgggtgtctgttttgg
BCV 700(-)	+	gcaggtcaatttgtgggttgggtt
BCV 750(-)	+	atcccggaaatttattgttccttg
BCV 770(-)	+	cttgggttatgtacttgcgtaagt
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BCV 900(-)	+	gggtaagttttctaagaaggctta
BCV 950(-)	+	ttaaaccgcttctctatgtagacc
BCV 979(-)	+	ggttgtgattatactggtgg
BCV 1114(-)	+	cgtgatccacgttatgttatg
BCV 1159(-)	+	cgtagtgttgcatatgttgc
BCV 1246(-)	+	gactctattattttacgtcaac
BCV 1302(-)	+	ggaggcagatgcagttgtaaatgc
BCV 1365(-)	+	gcagtttggttatattgactgc

a The negative symbols in the oligonucleotide names indicate the polarities of the nucleic acids to which the oligonucleotides anneal.

b Polarity of the oligonucleotide relative to the positive-strand viral genome.

Table S1. Oligonucleotides used in the study (*Continued*)

Oligonucleotides[a]	Polarity[b]	Sequence (5' to 3')
BCV 1402(-)	+	gactgcgaacaagacttgtgtg
BCV 1536(-)	+	gagtgcagcaggttatggtgg
BCV 2002(-)	+	ggtcaagcattttgtgactacg
BCV 23341(-)	+	ggatgctggttttactagc
BCV 23415(-)	+	ggtatgataatgttagcagtg
BCV 23498(-)	+	cctatttgtgtgtatgatcc
BCV 23730(-)	+	caccggtgctccttctattagcac
BCV 23953(-)	+	gctaaggtcaaaaataccaagg
BCV 24270(-)	+	ccatttttatcaagaaggtgg
BCV 24607(-)	+	cgacgtatacctaatcttcc
BCV 24647(-)	+	ggcttaatgataagtcggtgcccctc
BCV 24775(-)	+	gctgctaagatatatggtatg
BCV 25171(-)	+	ggtataggcacttgtcctgc
BCV 25310(-)	+	gcataggtgagcactgttcgg
BCV 25543(-)	+	gatctttatggtattacagg
BCV 26026(-)	+	ggtttgtatgaaattcaaatacc
BCV 26051(-)	+	cagagtttactataggtaatatggagg
BCV 26650(-)	+	gtgttaccatggatgtgttaagtc
BCV 26710(-)	+	gctcttgatgctattcaggaagg
BCV 27128(-)	+	ctactaagtatgtcactgcg
BCV 27505(-)	+	cagagctacatcaatctcaagg

a The negative symbols in the oligonucleotide names indicate the polarities of the nucleic acids to which the oligonucleotides anneal.

b Polarity of the oligonucleotide relative to the positive-strand viral genome.

Table S2. Oligonucleotides used for RT-qPCR

Oligonucleotides[a]	Polarity[b]	Sequence (5’ to 3’)
BCV 14427(-)	−	gctgctgatccagctttgcatgtagc
BCV 14588(+)	+	cagatcaactgaactaccctctttaagcagg
BCV 29397(-)	−	atgtcttttactcctggtaagcaa
BCV 29590(+)	+	gaaactgagtaattccagagaacc
H18s rRNA(-)	−	cattcgaacgtctgccctatc
H18s rRNA(+)	+	gcctgctgccttccttggatgtggtagcc

a The positive and negative symbols in the oligonucleotide names indicate the polarities of the nucleic acids to which the oligonucleotides anneal.

b Polarity of the oligonucleotide relative to the positive-strand viral genome.