FULL PAPER

Virology

The full genome sequences of 8 equine herpesvirus type 4 isolates from horses in Japan

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ABSTRACT.　Equine herpesvirus type 4 (EHV-4) is one of the most important pathogens in horses. To clarify the key genes of the EHV-4 genome that cause abortion in female horses, we determined the whole genome sequences of a laboratory strain and 7 Japanese EHV-4 isolates that were isolated from 2 aborted fetuses and nasal swabs of 5 horses with respiratory disease. The full genome sequences and predicted amino acid sequences of each gene of these isolates were compared with of the reference EHV-4 strain NS80567 and Australian isolates that were reported in 2015. The EHV-4 isolates clustered in 2 groups which did not reflect their pathogenicity. A comparison of the predicted amino acid sequences of the genes did not reveal any genes that were associated with EHV-4-induced abortion.

KEY WORDS:　abortion, genome sequence, Japanese equine herpesvirus type 4

Equine herpesviruses type 1 (EHV-1) and type 4 (EHV-4) cause equine respiratory disease (rhinopneumonia) in horses. EHV-1 and EHV-4 are classified as members of the genus *Varicellovirus* of *Alphaherpesvirinae*. The EHV-1 and EHV-4 genomes are linear, double-stranded DNA molecules of 150 kbp and 146 kbp in length, respectively [21, 22]. The genome structure of both EHV-1 and EHV-4 consists of a long unique region (UL) flanked by a short inverted repeat (TRL/IRL) linked to a short unique region (US) flanked by a substantial inverted repeat (TRS/IRS) [21, 22]. The genome of EHV-1 encodes 80 open reading frames (ORFs), while EHV-4 encodes 79 ORFs. EHV-1 and EHV-4 are genetically closely related viruses. The predicted amino acid sequences of their genes have identities in the range of 54.9–96.4% [22], and EHV-4 and EHV-1 are serologically similar. However, the symptoms of EHV-1 and EHV-4 are different: EHV-1 causes respiratory diseases in mostly 3-year-old horses and abortion in mares at the late stage of gestation [1, 15], while EHV-4 causes respiratory diseases in 2-year-old and under foals and rarely causes abortion [15, 22]. Other studies have shown that these differences are derived from the cellular tropisms of EHV-1 and EHV-4 [13]. However, it is unknown how these similar viruses have different pathogenicities in horses.

The full genome sequence of EHV-4 was first reported for an Irish isolate in 1998 [22]. Additional genome sequences of Australian isolates were reported in 2015 [24]. Maeda *et al.* reported the genomic variability among Japanese EHV-4 field isolates by restriction endonuclease digestion analyses and PCR to identify unique markers for the isolates [14]. Although EHV-4 is rarely associated with abortion in horses, at least 2 isolates from aborted fetuses were reported in Japan [14]. The incidence of abortion due to EHV-4 in the field is not well known, but it was reported to be less than 1% in Kentucky, U.S.A. between 1983 and 1992 and up to 16% in England between 1987 and 1993 [18, 26]. EHV-4 was also detected in a nasal swab of an aborted fetus by real-time PCR in Turkey [23].

In the present study, we determined the full genome sequences of EHV-4 isolates from 8 horses in Japan. To identify EHV-4

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genes associated with abortion and to establish diagnostic methods for predicting and preventing abortion, we compared the full genome sequences of isolates of respiratory associated isolates and abortion associated isolates from Japan, Ireland and Australia.

MATERIALS AND METHODS

Cells and viruses

Fetal equine kidney (FEK) cells were used to propagate viruses. The FEK cells were maintained in Dulbecco's Modified Eagle's Medium (D-MEM) (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) with 10% fetal bovine serum (FBS) (Invitrogen, Life Technologies, Tokyo, Japan).

Seven EHV-4s were isolated from aborted fetus in 1991 and 2012 (isolates 91c1 and 12-I-203, respectively) and nasal swabs of racehorses in 1983, 2001, 2003, 2005 and 2011 (isolates 83-MB, 01-10-1, 03-VR, 05-I-202 and 11-10, respectively). The isolate of 91c1 was kindly provided by Chubu Livestock Hygiene Service Center, Saga, Japan. A Japanese strain, TH20p, was plaque purified from TH20 which was isolated from a colt with respiratory disease in the first rhinopneumonitis outbreak in Japan was also used [14]. The other isolates and TH20p were kindly provided by Dr. Matsumura and Dr. Tsujimura (Japan Racing Association, Shimotsuke, Japan). The viruses were propagated in FEK cells for 2 passages, and supernatant was harvested when 80% CPE were observed.

Genome DNA extraction

The supernatant of a virus-infected cell culture was centrifuged at 3,000 rpm for 15 min at 4°C to remove cell debris and was ultracentrifuged at 25,000 rpm for 1 hr at 4°C to collect virions as a pellet. Viral genomic DNA was extracted from the pellet as described previously [25].

Sequencing and analysis of the genomes

Genome sequences of TH20p, 05-I-202 and 12-I-203 were read by the next generation sequencer (NGS) GS Junior (Roche Diagnostics K. K., Tokyo, Japan), and those of 83-MB, 91c1, 01-10-1 03-VR and 11-10 were read by the NGS MiSeq (Illumina lnc., Tokyo, Japan) according to the manufacturer's protocol. The complete genome sequences were assembled by reference sequence mapping with Bowtie 2 [10] using EHV-4 strain NS80567 sequence (GenBank accession number AF030027) or TH20p sequence as the reference and editing with Consed [4] and SnapGene software (GSL Biotech; available at snapgene. com). The full genome sequence of TH20p was determined by mapping against the NS80567 sequence. The genome sequences of the other 7 Japanese isolates were determined by mapping against TH20p as a reference sequence. Because ORFs 24 and 71 included large regions of direct repeat sequences, these regions were amplified by PCR and further analyzed by Sanger sequencing. The primers used to amplify the ORFs 24 and 71 were ORF24-F (5′-gctttccaaaccttggcgtccatcgatacg-3′), ORF24-R (5′-ccgcgcggtttatctgtagatcatattcaagttc-3′), ORF71-F (5′-ctacatcaacctcggtgtc-3′) and ORF71-R (5′-cctgtttgatccaaccgacc-3′). Direct repeat sequences were analyzed by Tandem Repeat Finder [3] and XSTREAM [16] and removed from sequences before multiple alignments. The locations of removal are indicated in Table 1. The whole genome, UL, US and IR of EHV-4s including sequences determined in the present study, and EHV-4 reference strain NS80567 and 14 sequences of Australian isolates [24] were multiple aligned by MAFFT version7 [7] and AliView 1.18 [11]. Phylogenetic trees were constructed by neighbor-joining method using the Molecular Evolutionary Genetic Analysis (MEGA7) software [9] based on the nucleotide sequences excluding direct repeat sequences. Each ORF's amino acid (AA) sequence was analyzed as the same as nucleic acid sequences. The AA differences were counted using AliView 1.18 manually.

Nucleotide sequence accession numbers

The 8 EHV-4 genome sequences were submitted to DNA Data Bank of Japan with the following accession numbers: LC063142 (TH20p), LC075582 (83-MB), LC075583 (91c1), LC075584 (01-10-1), LC075585 (03-VR), LC075586 (05-I-202), LC075587 (11-10) and LC075588 (12-I-203).

RESULTS

The EHV-4 isolates consisted of 2 genetic groups

We determined full genome sequences of 8 Japanese EHV-4 isolates. The sizes of the EHV-4 full-length genomes ranged from 143,645 bp (91c1) to 144,802 bp (TH20p) (Table 2). Each virus genome encoded 79 of open reading frames (ORFs) as well as EHV-4 strain NS80567.

Next, we compared the genome of 8 Japanese isolates with other 15 EHV-4 isolates from Ireland and Australia. The phylogenetic trees were constructed by MEGA7 based on the nucleotide sequences excluding direct repeat sequences (Fig. 1a–1d). In the phylogenetic trees, the EHV-4 isolates fell into 2 genetic groups, groups I and II. Group I consisted of 13 isolates, and group II consisted of 10 isolates. All but one of the abortion isolates belonged to group I, the exception being 475-78 in group II.

Group II strains had a higher sequence similarity than group I strains. The structure of the UL tree (Fig. 1b) was very similar to that of the whole genome tree (Fig. 1a). Although the grouping in the US tree (Fig. 1c) was the same as that in the whole genome tree, the structures of the 2 trees were different. The variation was greatest in the IR region (Fig. 1d) which has higher GC mol% than in UL and US. GC mol% of ORFs and non-coding regions in IR are 66.7% and 53%, respectively.

Table 1. The deleted repeats location and size of NS80567 for alignment

Location	Size (bp)	Location	Size (bp)
100..507	408	122,168..122,311	144
44,157..44,696	540	127,846..127,905	60
44,763..44,906	144	127,906..128,205	300
45,048..45,095	48	128,261..128,344	84
73,525..73,775	251	135,725..135,868	144
108,448..108,735	288	138,102..138,281	180
112,550..112,564	15	145,135..145,446	312
112,583..112,894	312	145,468..145,482	15
119,751..119,930	180		

Table 2. EHV-4 field isolates sequenced in this study

Field isolates	Isolation site	Disease	Year of isolation	Accession number	Genome size (bp)
TH20p	Nasal swab	Respiratory disease	1962	LC063142	144,802
83-MB	Nasal swab	Respiratory disease	1983	LC075582	144,292
91c1	Aborted fetus	Abortion	1991	LC075583	143,645
01-10-1	Nasal swab	Respiratory disease	2001	LC075584	144,636
03-VR	Nasal swab	Respiratory disease	2003	LC075585	144,108
05-I-202	Nasal swab	Respiratory disease	2005	LC075586	144,655
11-10	Nasal swab	Respiratory disease	2011	LC075587	144,670
12-I-203	Aborted fetus	Abortion	2012	LC075588	143,996

The structures of ORFs 24 and 71 containing direct repeat sequences

To see if differences in the direct repeat sequences were related to viral pathogenicity, we analyzed the predicted AA sequences of ORF 24 in UL and ORF 71 in US. We searched direct repeat regions of AA sequence by XSTREAM, and other regions were recognized as unique sequence regions.

ORF 24, which is the largest tegument protein of EHV-4, has 3 direct repeat regions (regions II, IV and VI) and 4 unique sequence regions (regions I, III, V and VII) (Fig. 2). The lengths of regions I, III, V, VI and VII were the same among the isolates analyzed in this study. All the isolates had the same number of copies in region VI, while they had different number of copies in regions II and IV (Fig. 2). In region II, 7 isolates had less copy numbers and tended to include abortion isolates. Japanese isolates had more variations in region IV, one Japanese isolate had less than 13 copies, and 2 Japanese isolates had more than 13 copies.

ORF 71, which is a large glycoprotein, has 6 regions which contains 2 direct repeat regions (regions III and V) and 4 unique sequence regions (regions I, II, IV and VI) (Fig. 3). All the isolates analyzed in the study had the same number of AAs in regions I and IV, while they had significantly different numbers of copies in regions III and V and significantly different patterns of direct repeat unit sequences (Fig. 3). The length and sequence pattern of region II were different among the isolates. We focused on direct repeat regions to find variation of AA sequence of ORF 71, but the sequence variation was seen in region II.

The predicted AA sequences for both ORFs 24 and 71 varied among the isolates, however, abortion isolates did not have common feature of sequence patterns. These results indicate that neither of ORF was related to the pathogenicity of EHV-4.

Comparison of AA sequences of individual viral genes

To elucidate the genes that might be related to the pathogenicity of EHV-4, the predicted AA sequences of all 76 ORFs were compared among 23 EHV-4 isolates and strains (Table S1). Nine ORFs, including ORFs 4, 6, 8, 11, 25, 26, 44, 51 and 58, were completely conserved among the sequences examined. The predicted AA sequences for other ORFs varied among the isolates, as well as ORFs 24 and 71, and none of the ORFs seemed to correlate with pathogenicity of EHV-4 in horses.

DISCUSSION

In the present study, we determined the full genome sequence of 1 laboratory strain, TH20p and 7 Japanese isolates. Phylogenetic analyses were performed on a total of 23 isolates and strains of EHV-4 including the 8 Japanese strain and isolates, NS80567 (the Irish reference strain) and 14 Australian isolates. They clustered into 2 groups, I and II. The genome sequences of the 23 isolates and strains appeared to be very similar, especially in group II. TH20p is the oldest EHV-4 in this study, which was isolated in 1962 and maintained in laboratories in Japan. The newest Japanese isolate in group II, 05-I-202, was isolated in 2005. TH20p and 05-I-202 showed the highest similarity among Japanese isolates. This high similarity suggests that these 2 viruses have the same ancestor and have been conserved in horses in Japan. In phylogenetic tree analysis of EHV-4 genome, we found that IR region had the most variation among isolates. Natural recombination of viral genome is being recognized as important mechanism
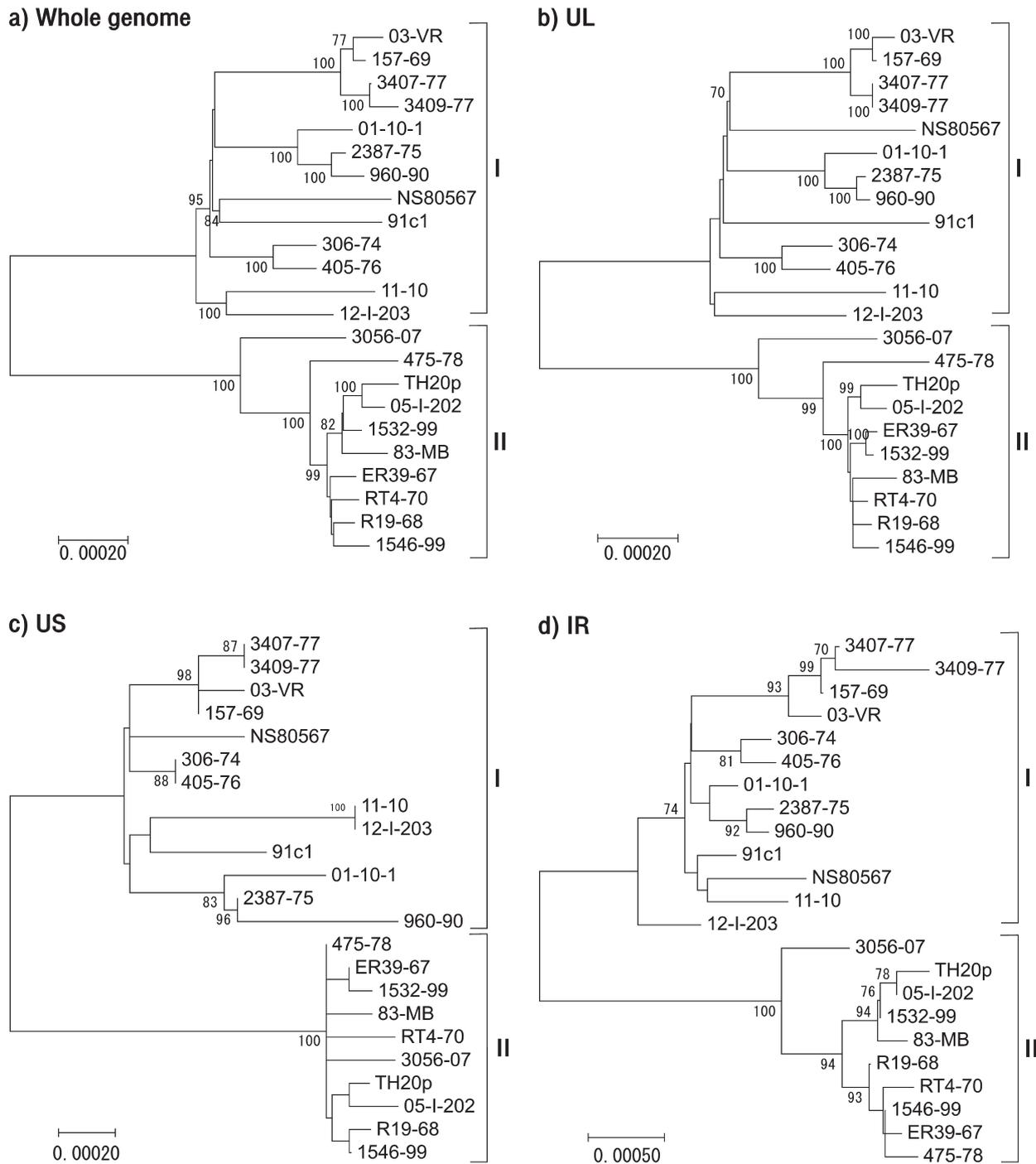
Fig. 1. Phylogenetic trees indicating evolutionary relationships generated from Japanese, Irish and Australian EHV-4 nucleotide alignments (excluding sequence repeats) using MEGA7. (a) Whole genome sequence, (b) UL region, (c) US region and (d) IR region. In all region, isolates and strains were clustered into group I or II. These groups were not associated with viral pathogenicity.

in alphaherpesviruses including EHV-4 [12, 24]. We did not examine recombination frequency of the EHV-4 genome in this study, however, a higher GC content in the repeats may increase their recombination frequency [12]. This indicates that higher mutation rate in high GC mol% of EHV-4 IR may contribute to recombination of viral genome in hosts to adapt them.

When we completed sequencing of EHV-4 isolates and the laboratory strain isolated in Japan, Australian group reported the genome sequences of Australian EHV-4 isolates [24]. Therefore, we analyzed the sequences of Japanese EHV-4 adding with those of Australian EHV-4 in the present study. We newly found that Japanese, Irish and Australian isolates were clustered into either the cluster I or II, indicating that genomic diversity of EHV-4 did not depend on geographical origins. This result suggested that globally distributed EHV-4 is clustered into the cluster I or II. To confirm this hypothesis, more EHV-4 sequences from other
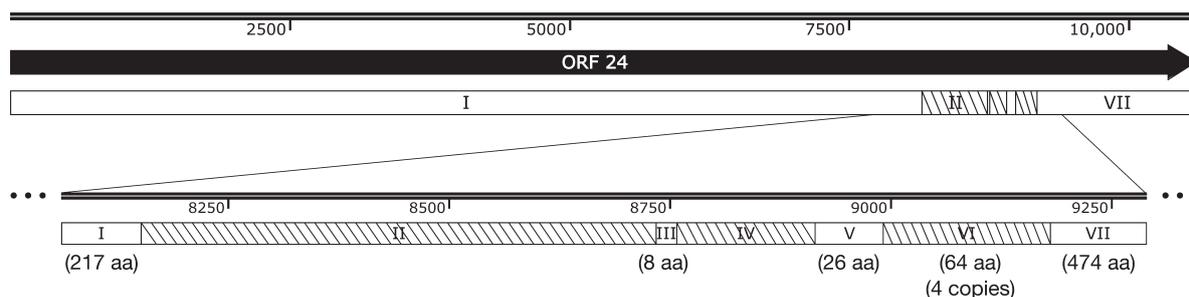
Virus strains and isolates	No. of copies			Virus strains and isolates	No. of copies	
	II	IV			II	IV
	APSKPAAAP	AKDQ			APSKPAAAP	AKDQ
91c1*	3.56	18		NS80567	21.56	13
12-I-203*	3.56	13		ER39-67	21.56	13
2387-75*	17.56	13		R19-68	21.56	13
3409-77*	10.56	13		157-69	21.56	13
475-78*	21.56	13		RT4-70	21.56	13
				306-74	21.56	13
TH20p	21.56	13		405-76	21.56	13
83-MB	4.56	13		3407-77	21.56	13
01-10-1	21.56	18		960-90	21.56	13
03-VR	2.56	10		1532-99	21.56	13
05-I-202	21.56	13		1546-99	10.56	13
11-10	21.56	13		3056-07	21.56	13

*: Isolated from aborted fetus

Fig. 2. Amino acid sequence structure of ORF 24. ORF 24 was divided into 7 regions indicated in the schematic diagram above, and the details of numbers of copied sequences and amino acid sequences were shown in the table below. Regions I, III, V and VII were not repeat sequences. Region II, IV and VI were direct repeat sequences. The ORF 24 sequence was highly conserved, except for 2 regions, Regions II and IV, and those were repeat sequences.
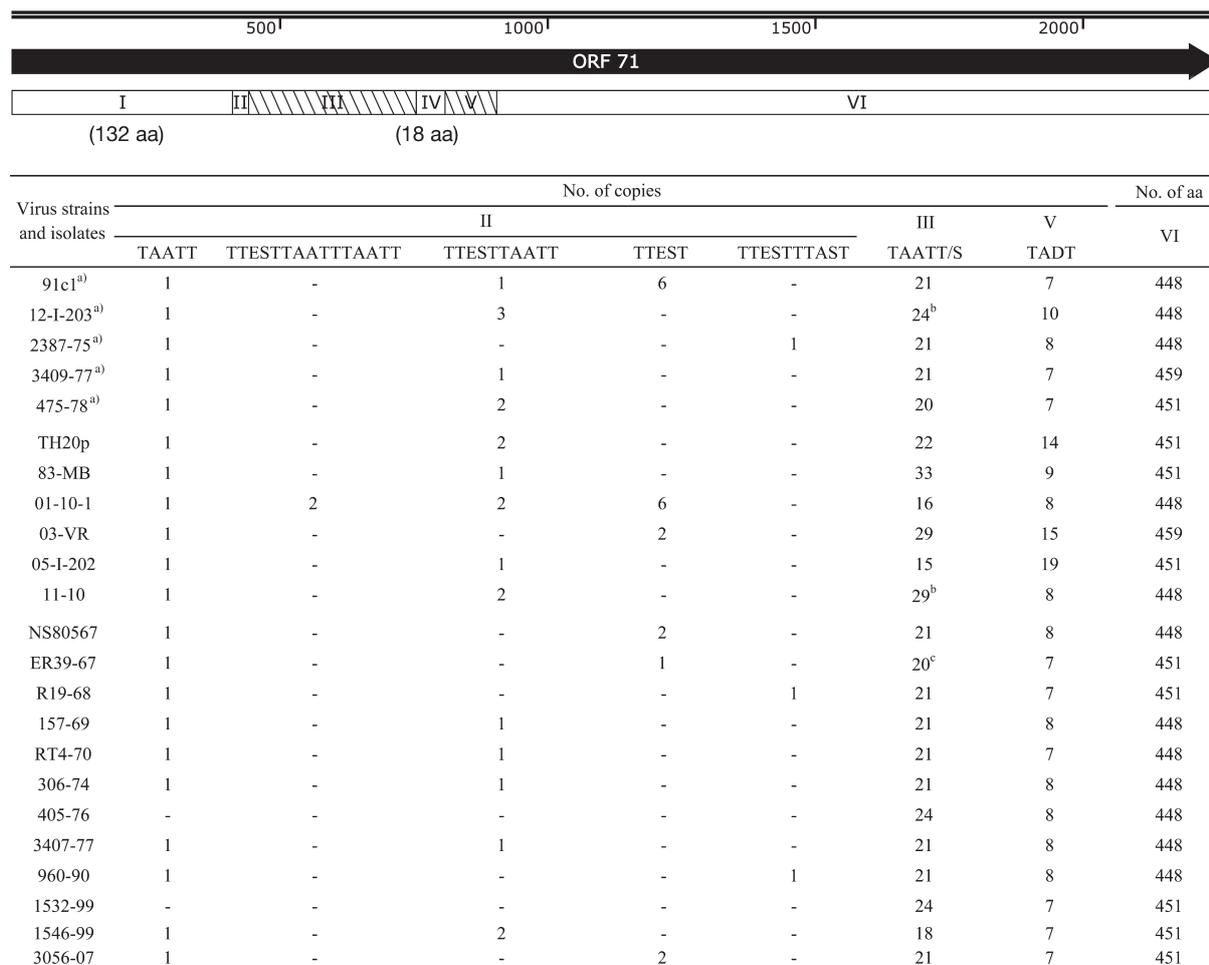
regions should be analyzed.

It is reported that repeat sequences of ORFs 24 and 71 were variable among EHV-4 field isolates [5, 14]. A large number of alterations were expected in ORFs 24 and 71, because these genes contain repeat regions. Although ORF 24 sequence is longer than ORF 71 sequence, it was revealed that ORF 24 had less repeat patterns and mutations compared to those of ORF 71. EHV-1 ORF 71, which has 61.1% identity with EHV-4 ORF 71 [22], is reported to be sufficient to restore respiratory virulence to the attenuated KyA strain [19].

The economic loss due to abortion caused by EHV-1 and 4 is a severe problem in the horse industry. It is believed that the mechanism of abortion by EHV-1 and 4 is similar but the pathogenesis of EHV-4 has not been well studied [18]. The neuropathogenicity of EHV-1 is determined by an AA change of ORF 30 (D752N) [17]. We hypothesized that the molecular marker related to abortion would be clear in EHV-4 because abortion rarely happens in EHV-4. However, we could not find the key gene (s) of abortion in the present study.

We consider the reasons of failure to identify the key gene (s) associated with abortion by EHV-4 as follows. First, the mechanism of abortion caused by EHV-1 or EHV-4 is complicated, for instance, not only one gene or SNP might determine the viral pathogenicity. Second, not only the viral factors but also host factors are critical to induce abortion. Third, the viral passages in culture cells derived from unnatural species induce mutations in the EHV-4, because mutations associated with viral passages in culture cells were observed in EHV-1 genomes [2, 6, 8, 20]. In conclusion, to solve these problems, we need to collect more viral and host samples to compare the genomic diversity statistically between respiratory associated isolates and abortion associated isolates. Furthermore, DNA sample preparation for next generation sequence should be done directly from specimen in a future study to avoid detecting mutations derived from viral passages in culture cells.

| Virus strains and isolates | No. of copies | | | | | | | No. of aa |
| | II | | | | | III | V | VI |
	TAATT	TTESTTAATTTAATT	TTESTTAATT	TTEST	TTESTTTAST	TAATT/S	TADT	
91c1[a]	1	-	1	6	-	21	7	448
12-I-203[a]	1	-	3	-	-	24[b]	10	448
2387-75[a]	1	-	-	-	1	21	8	448
3409-77[a]	1	-	1	-	-	21	7	459
475-78[a]	1	-	2	-	-	20	7	451
TH20p	1	-	2	-	-	22	14	451
83-MB	1	-	1	-	-	33	9	451
01-10-1	1	2	2	6	-	16	8	448
03-VR	1	-	-	2	-	29	15	459
05-I-202	1	-	1	-	-	15	19	451
11-10	1	-	2	-	-	29[b]	8	448
NS80567	1	-	-	2	-	21	8	448
ER39-67	1	-	-	1	-	20[c]	7	451
R19-68	1	-	-	-	1	21	7	451
157-69	1	-	1	-	-	21	8	448
RT4-70	1	-	1	-	-	21	7	448
306-74	1	-	1	-	-	21	8	448
405-76	-	-	-	-	-	24	8	448
3407-77	1	-	1	-	-	21	8	448
960-90	1	-	-	-	1	21	8	448
1532-99	-	-	-	-	-	24	7	451
1546-99	1	-	2	-	-	18	7	451
3056-07	1	-	-	2	-	21	7	451

[a] Isolated from aborted fetus. [b] The repeat sequence of TTEST was included in the region. [c] The repeat sequence of PQLLL was included in the region.

Fig. 3. Amino acid sequence structure of ORF 71. ORF 71 was divided into 6 regions indicated in the schematic diagram above, and the details of numbers of copied sequences and amino acid sequences were shown in the table below. Regions I, II, IV and VI were not repeat sequences, but region II partially included repeat sequence. Regions III and V were direct repeat sequences which determined the length of ORF 71 amino acid sequence. Regions II and III were the most variable region.

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