Isolation of equine herpesvirus 3 (EHV-3) from equine coital exanthema of two stallions and sero-epidemiology of EHV-3 infection in Japan

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ABSTRACT. In the spring of 2015, two stallions reared in Farms A and B in Hokkaido in Japan showed symptoms of equine coital exanthema. Equine herpesvirus 3 (EHV-3) was isolated from penis swab samples of both stallions, and the isolates from each stallion in Farms A and B were designated as SS-1 and YS-1 strains, respectively. *Bam*HI restriction profiles of SS-1 and Japanese reference strain Iwate-1 were indistinguishable, but the *Bam*HI-A fragment of YS-1 was larger than those of SS-1 and Iwate-1 by 1.9 kbp because of the lack of two *Bam*HI sites. Nucleotide sequence analyses of glycoprotein G (gG), gB, gC and VP13/14 coding regions revealed that SS-1 and YS-1 had 99.77% to 100% identities to each other. These results suggested that the origins of SS-1 and YS-1 were different. For a sero-epidemiological survey, serum neutralizing tests using SS-1 against 319 sera of horses from eight farms in Hokkaido were conducted. Six of the eight farms were EHV-3 antibody-positive, and positive rates ranged from 2.6% to 17.6%. To determine the infection time of four EHV-3 antibody-positive horses, a retrospective study was conducted. Infection time of the four horses was in the breeding season, and re-infection or reactivation of latently infected EHV-3 might have occurred in one horse. However, these four horses had never shown any clinical symptoms. The results suggested that several EHV-3 strains are distributed in Japan and that infection is maintained widely in horses without clinical symptoms.

KEY WORDS: ECE, EHV-3, isolation, sero-epidemiology, stallion

Equine coital exanthema (ECE) caused by equine herpesvirus 3 (EHV-3) is an acute, infectious, predominantly venereally transmitted disease, resulting in the formation of papules, vesicles, pustules and ulcers on the penis and prepuce of stallions and on the vaginal and vestibular mucosa and perineal skin of mares [3]. Pathological lesions caused by non-coital infection through infected fomites and iatrogenic transmission have occasionally been observed on the skin of the lips and mucous membrane of the respiratory tract [4, 13, 21, 32]. Many infections are subclinical or mild. EHV-3 does not cause viremia and is non-abortigenic under natural conditions. EHV-3 is restricted to the epithelium of the nasal and vaginal mucosae, and the virus neither breaches the basement membrane nor infects individual immune cells [23]. EHV-3 is a member of the order *Herpesvirales*, family *Herpesviridae*, subfamily *Alphaherpesvirinae* and genus *Varicellovirus* [1]. EHV-3 showed no serologic cross-reactivity with other equine herpesviruses in neutralization tests [3, 17, 28]. Recently, the complete EHV-3 genomic sequence has been reported. It has a total size of 151,601 nucleotides and encodes 76 distinct genes [27]. EHV-3 is highly host-specific and has been grown only in cells of equine origin [11]. EHV-3 might establish a latent infection in an undefined site, from where reactivation and shedding might occur [1, 5, 6, 12, 29]. New outbreaks of disease might occur by reactivation of latent virus in clinically normal infected carrier animals [5, 29].

EHV-3 was first isolated in 1968 concurrently in the U.S.A., Canada and Australia [10, 16, 25] and has been shown to be distributed worldwide [3]. In Japan, although symptoms similar to those of ECE have occasionally been observed, the disease is obviously underdiagnosed, either because it is mild or because virological examinations at appropriate times are not performed. There has been only one report concerning isolation of EHV-3 from affected horses in Japan [26]. In this report, isolation of EHV-3 from two stallions affected with ECE that were reared in different farms and molecular characterization of the isolated

viruses are described. Furthermore, by using one of the isolated viruses, a sero-epidemiological survey was conducted in horses in Hokkaido, Japan.

MATERIALS AND METHODS

Cell culture and virus

Fetal horse kidney (FHK) cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum and used for virus isolation and serum neutralization tests. For the maintenance medium, the serum concentration was reduced to 4%. The Iwate-1 strain was propagated in FHK cells and used as the reference strain of EHV-3 in this study [26].

Samples from affected horses

Stallion A at Farm A and stallion B at Farm B developed ECE clinically on April 10, 2015 and May 14, 2015, respectively. The day after the onset, swabs were obtained from penile lesions of both stallions and placed in 1 ml of MEM supplemented with 50 μg/ml of gentamycin. The eluted samples were centrifuged at 670 × g for 10 min at 4°C to pellet debris, and the supernatant fluids were used for virus isolation and DNA extraction. Paired sera were collected on April 11 and 27 from stallion A and on May 15 and June 3 from stallion B.

Virus isolation

One hundred μl of each of the supernatant fluids of swab samples was inoculated onto FHK cells cultured in a 6-well plate. After adsorption at 37°C for 30 min under a 5% CO_2 atmosphere, the cells were washed twice with MEM, and the maintenance medium was added. Then, the cells were cultured at 37°C under a 5% CO_2 atmosphere.

DNA extraction: DNA was extracted from virus-infected cells as previously described [18]. Viral DNA was extracted from the supernatant fluids of swab samples described above and from isolated viruses in cell-cultured fluids using a DNeasy Blood and Tissue kit (Qiagen K.K., Tokyo, Japan).

Restriction enzyme digestion

The DNAs were digested to completion with restriction endonuclease *Bam*HI under conditions recommended by the manufacturer (Takara Bio Inc., Tokyo, Japan). The digested fragments were separated on 0.7% agarose gels as previously described [20].

Polymerase chain reaction (PCR) for detection of EHV-3

To detect EHV-3 DNA in swab samples, isolated virus and the reference virus, PCR assays targeting the gG gene were carried out using Expand High Fidelity PCR System (Roche Diagnostics GmbH, Mannheim, Germany) and primers listed in Table 1 as described by Dynon *et al.* [14]. The PCR products were purified and sequenced as described below.

Sequencing

Regions coding for gB, gC and VP13/14 and partial terminal regions of *Bam*HI-A and adjacent fragments were amplified by PCR with KOD-Plus-Neo (Toyobo Co., LTD., Osaka, Japan) and each of the specific primers listed in Table 1. The PCR products were purified with a High Pure PCR Product Purification kit (Roche Diagnostics GmbH) or with a QIAquick Gel Extraction kit (Qiagen K.K.), if extra bands were observed. The purified PCR products were used for sequencing. Sequencing was conducted in Hokkaido System Science Co., Ltd. (Sapporo, Japan) using the specific primers and walking primers. Sequence analyses were conducted by DNASIS Pro (Hitachi Software Engineering Co., Ltd., Tokyo, Japan). DDBJ accession numbers assigned to the gene sequences of the analyzed isolates and Iwate-1 strain are LC189056-LC189067, LC189551-LC189554 and LC199967-LC199968.

Serum samples

For a sero-epidemiological survey, sera from eight farms (A to H) in Hokkaido were used. Farms A and B were stud farms, and the others were breeding farms. Sera were collected from 44 stallions in Farm A and 17 stallions in Farm B on April 27, 2015 and June 3, 2015, respectively. Additionally, sera were collected from 117 mares in Farm C, 39 mares in Farm D and 30 mares in Farm E on April 29, 2015, February 10, 2014 and January 13, 2015, respectively. Sera were also collected from 30 mares in Farm F, 25 mares in Farm G and 17 mares in Farm H on March, in 2014, January 26, 2015 and October 26, 2011, respectively. When a horse in Farm A or C was considered positive by serology, we used elapsed sera that had been stocked in our laboratory since 2002 to estimate the EHV-3 infection time.

Serum neutralization (SN) test

Antibodies against EHV-3 in sera were determined by the SN test using the SS-1 isolate of this study and FHK cells as described previously [26].

RESULTS

Virus isolation and identification

ECE was strongly suspected, because of the clinical symptoms observed in the two affected stallions, A and B, in Farm A and

Table 1. Primers used in PCR amplification

Target regions	Primer	Sequence	Location	PCR product size (bp)
gG gene	gG-F	GCGCTCTCTCGGCCTTGCCAG	132949-132969[a)]	518
	gG-R	GGCGTCTCGAAAAGCGAGAG	133466-133447	
gB gene	gB-F	TTCTCCTCGGTTTTCCACTG	60484-60503	3,159
	gB-R	TGTCGGATACGCGTAATGTT	63642-63623	
gC gene	gC-F	TAATCGAGATCGGCGAGTTG	20328-20347	1,531
	gC-R	GCACGAAACCCTGTTTGC	21858-21841	
VP13/14 gene	13-F	TGCGCTTTCGTCTGTGATAC	14285-14304	2,766
	13-R	GGGTAGAGGCGCACAAAG	17050-17032	
*Bam*HI-A	A-F	AAGAGGAGTGTAAGCGAAAGGA	125328-125349	1,180
			143754-143733	1,180
	A-R	TAGCCCATCGCGTAGAAATC	126507-126488	
			142575-142594	

a) Location at the complete nucleotide sequence of EHV-3 strain AR/2007/C3A (Genbank KM051845.1).

|			
Iwate-1	813	GGGCCCTCAATCCCTGCTCA TTGGCGCCCTGGGACTTCGC ATCCTGAGCCAGCGCTGGTA CATGCTGCCGGGCGAGACGT ACGACCAGCT	902
SS-1	813	....A....	902
YS-1	813	....A....	902
AR/2007/C3A	813	....A....	902
Iwate-1	903	GAGGCAAAATTCCAGAGGGT CTGCCCGCGGCGCAGACAGG GAATCCGCGGCCGACGTAAC AGAACCCGAAGAAAAGCCTT CGGAAAAAAC	992
SS-1	903	.C..................	992
YS-1	903	....	992
AR/2007/C3A	903	.C..................	992
Iwate-1	993	CCCCGCTTCTCCCACCGATG ACGAGGAGAAAGAAGAAGAG GAAAACGGGGATAACGAGCC AACCCCAGCGCCACCGGCCC CGGGATGCGA	1082
SS-1	993	....	1082
YS-1	993	....	1082
AR/2007/C3A	993	....	1082
Iwate-1	1083	CGAGCAAGACGCACCTGCCG CCGGAGACGGATCTCCGTGG TACACCGGCGGCATTCTCGT GTCCGGTCCTGAGTGCGGAC AGCAGAAGGG	1172
SS-1	1083	....A..............	1172
YS-1	1083	....	1172
AR/2007/C3A	1083	....A..............	1172
Iwate-1	1173	GACCAACTACGCGGGTATCG GCTTTCTCATCTTGGGAGTG TGTCTCCTCATCGGCCTCAT TGTCTACGTTTGCGTCCTGC GGTCCAGAGT	1262
SS-1	1173	....	1262
YS-1	1173	....	1262
AR/2007/C3A	1173	....	1262
Iwate-1	1263	CTCCGAGCGCAAGCTCCACA ACAGCTACTCTCGCTTTT	1300
SS-1	1262	.G.................	1300
YS-1	1262	....	1300
AR/2007/C3A	1263	.G.................	1300

Fig. 1. Comparison of nucleotide sequences of the partial gG gene from two field strains (SS-1 and YS-1) with those of Japanese reference strain Iwate-1 and Argentina AR/2007/C3A strain. The identical nucleotide is indicated by a dot. Numbers on the left and right sides are the nucleotide position of the EHV-3 gG gene.

Farm B, respectively [31]. Cytopathic effects (CPE) were observed in FHK cells after one-day post inoculation of penis swab samples from both stallions. CPE of both isolates were characterized by rounded cells and syncytial formation (data not shown). The isolates from stallions A and B were designated as SS-1 and YS-1 strains, respectively. By PCR amplification of the partial gG gene of EHV-3, identical products with the same estimated size, approximately 520 bp, were detected from DNAs of penis swab
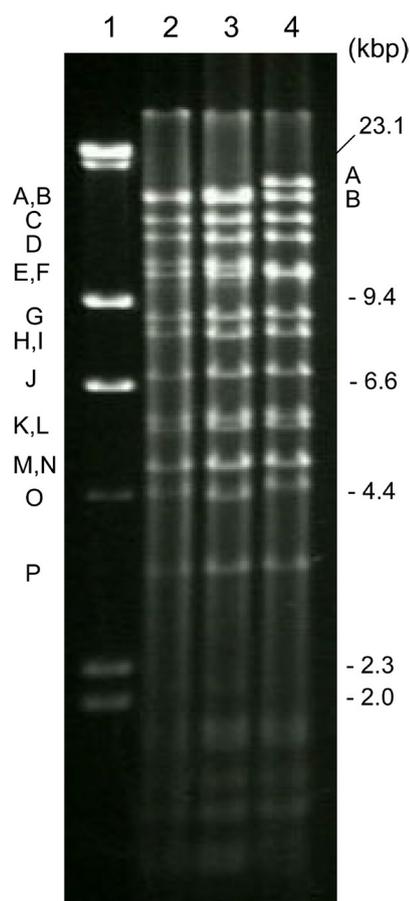
Fig. 2. *Bam*HI restriction profiles of DNAs of SS-1 and YS-1 strains and Japanese reference strain Iwate-1. Lane 1: molecular weight marker, lambda DNA *Hind* III digest, Lane 2: Iwate-1, Lane 3: SS-1, Lane 4: YS-1.
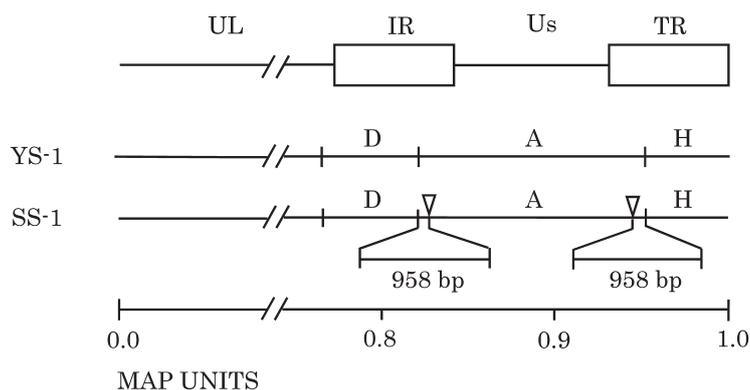
Fig. 3. *Bam*HI partial restriction map of SS-1 and YS-1 strains. The map was based on Sullivans *et al.* [30]. Internal repeat (IR) and terminal repeat (TR) are indicated by open boxes. Extra *Bam*HI sites observed in SS-1 are indicated by open triangles.

Table 2. EHV-3 neutralizing antibody titers in stallions A and B

Stallion	Serum collection dates in 2015	
	April 11	April 27
A	<2	4
	May 15	June 3
B	<2	2

samples of both stallions and isolated viruses (data not shown). The nucleotide sequence identities of the amplified products of SS-1 and YS-1 were 99.18% and 99.80%, respectively, against that of the Japanese reference strain Iwate-1 and 100% and 99.39%, respectively, against that of full-genome sequenced Argentina isolate AR/2007/C3A (Fig. 1). *Bam*HI restriction profiles revealed that SS-1 and Iwate-1 showed almost the same pattern (Fig. 2). A remarkable difference between SS-1 and YS-1 profiles was observed in the *Bam*HI-A fragments. The *Bam*HI-A fragment of YS-1 was larger than those of SS-1 and Iwate-1 by approximately 1.8 kbp. To clarify the size difference in *Bam*HI-A fragments between SS-1 and YS-1, we conducted sequence analysis on partial terminal regions of *Bam*HI-A fragments. Based on the *Bam*HI restriction map [30], the *Bam*HI-A fragment consists of a unique short (Us) segment bracketed by a part of the inverted repeat sequences (Fig. 3). PCR primers were designed within the repeat sequences to amplify the estimated size of 1.2 kbp of both terminals of the *Bam*HI-A fragment of YS-1. These primers could also amplify the same size of PCR products in SS-1. Sequence analysis of both PCR products revealed that one *Bam*HI restriction site, which was located at 958 bp from the 5′ end of the SS-1 PCR products, was missing in the YS-1 PCR product (Fig. 3). Since this site was within both inverted repeat sequences, the size difference between SS-1 and YS-1 *Bam*HI-A fragments was 1,916 bp. From these results, SS-1 and YS-1 were identified as EHV-3, but *Bam*HI profiles of the two viruses were slightly different. The EHV-3 neutralizing antibody titers of paired sera of stallions A and B are shown in Table 2. In convalescent sera, EHV-3 antibodies were detected in both stallions with low titers. Both stallions were treated by administering valacyclovir, an anti-herpesvirus agent, orally from the day after onset for 10 days in stallion A and from the date of occurrence for 9 days in stallion B [31].

Sequence analysis

SS-1 and YS-1 were isolated at almost the same time in the same prefecture. In order to determine whether SS-1 and YS-1 were similar or not, complete nucleotide sequences of gB, gC and VP13/14 were determined and compared, and the sequences were also compared to those of Iwate-1 and Argentina isolate AR/2007/C3A. The gB nucleotide sequences and deduced amino acid sequences of SS-1, YS-1 and Argentina isolate were the same, 100% identities, but not identical to those of Iwate-1, 99.77% and

Table 3. Identities of nucleotide sequence and amino acid sequence of gB among EHV-3 isolates

| | SS-1 | Identity (%) of nucleotide sequence (2,982 bp) | | |
		YS-1	Iwate-1	AR/2007/C3A[a]
SS-1		100.00	99.77	100.00
YS-1	100.00		99.77	100.00
Iwate-1	99.80	99.80		99.77
AR/2007/C3A	100.00	100.00	99.77	
		Identity (%) of amino acid sequence (994 aa)		

a) Argentina isolate (KM051845.1).

Table 4. Identities of nucleotide sequence and amino acid sequence of gC among EHV-3 isolates

| | SS-1 | Identity (%) of nucleotide sequence (1,419 bp) | | |
		YS-1	Iwate-1	AR/2007/C3A[a]
SS-1		99.93	99.79	99.79
YS-1	99.79		99.86	99.86
Iwate-1	99.58	99.79		99.72
AR/2007/C3A	99.79	100.00	99.79	
		Identity (%) of amino acid sequence (473 aa)		

a) Argentina isolate (KM051845.1).

Table 5. Identities of nucleotide sequence and amino acid sequence of VP13/14 among EHV-3 isolates

| | SS-1 | Identity (%) of nucleotide sequence (2,658 bp) | | |
		YS-1	Iwate-1	AR/2007/C3A[a]
SS-1		99.89	99.81	99.92
YS-1	99.77		99.85	99.81
Iwate-1	99.66	99.89		99.74
AR/2007/C3A	100.00	99.77	99.66	
		Identity (%) of amino acid sequence (886 aa)		

a) Argentina isolate (KM051845.1).

99.80% identities, respectively (Table 3). The gC nucleotide sequence and deduced amino acid sequence of SS-1 were not identical to those of the other three viruses, 99.79% to 99.93% identities and 99.58% to 99.79% identities, respectively (Table 4). VP13/14 nucleotide sequences and deduced amino acid sequences of SS-1 and YS-1 were not identical, 99.89% and 99.77% identities, respectively (Table 5). The results showed that SS-1 and YS-1 were similar isolates and also similar to Iwate-1 and Argentina isolate.

Sero-epidemiology

To examine the prevalence of EHV-3 antibody in Japanese horses, we conducted SN tests using SS-1 strain and a total of 319 sera from the two stallion farms (Farms A and B) and six broodmare farms (Farms C to H) (Table 6). In Farms A and B, positive rates were 9.1% and 17.6%, respectively. Three of the six broodmare farms showed positive rates ranging from 2.6% to 3.4%. The positive rate for stallions (11.5%) was higher than that for broodmares (2.3%). To estimate the EHV-3 infection time of EHV3 antibody-positive horses, we conducted a retrospective study in three stallions in Farm A and one broodmare in Farm C (Table 7). Our laboratory has been stocking sera from horses in Farms A and C since 2002. Stallion No. 411 showed sero-conversion in June 2011 and May 2014. Stallion No. 825 showed sero-conversion in August 2013. Stallion No. 833 showed sero-conversion in May 2013. Broodmare No. 263 showed sero-conversion in May 2012. These four horses showed sero-conversion in the breeding season.

DISCUSSION

In this study, we isolated two EHV-3 strains from separate ECE cases of two stallions in Japan. Sero-conversion against EHV-3 in convalescent sera of both stallions supported their EHV-3 infection. However, EHV-3 neutralizing antibody titers in the convalescent sera were low. Some studies have shown that peak antibody titers against EHV-3 were relatively low compared

Table 6. Prevalence of EHV-3 neutralizing antibody in horses in Hokkaido in Japan

Farm	Category	Collecting date	Positive sera/tested sera
A	Stallion	27 Apr. 2015	4[a] (4 - 16)[b]/44 (9.1)[c]
B	Stallion	3 Jun. 2015	3[d] (2 - 16)/17 (17.6)
C	Broodmare	29 Apr. 2015	4 (2 - 8)/117 (3.4)
D	Broodmare	10 Feb. 2014	1 (4)/39 (2.6)
E	Broodmare	13 Jan. 2015	0/30 (0)
F	Broodmare	11 Mar. 2014	1 (8)/30 (3.3)
G	Broodmare	26 Jan. 2015	0/25 (0)
H	Broodmare	26 Oct. 2011	0/17 (0)
Subtotal	Stallion		7/61 (11.5)
	Broodmare		6/258 (2.3)
Total			13/319 (4.1)

a) One positive serum is from stallion A. b) Numbers in parenthesis are the range of EHV-3 neutralizing antibody-positive titers. c) Number in parenthesis is positive percent. d) One positive serum is from stallion B.

Table 7. Retrospective study of EHV-3 neutralizing antibody positive-horses in Farms A and C

Farm	Horse No.	Neutralizing antibody titer															
		'02[a]	'07	'08	'09	'10	'11				'12	'13	'14				'15
		7[b]	7	7	7	10	5	6	7	8	6	6	4	5	7	12	4
A	411 (♂, 23y)[c]	-[d]	-	-	-	-	-	8	2	-	-	-	-	32	16	8	16

		'10	'11	'12	'13				'14	'15
		10	7	10	6	7	8	9	12	4
	825 (♂, 8y)	-	-	-	-	-	4	4	8	4

		'11	'12	'13			'14	'15
		7	10	4	5	6	12	4
	833 (♂, 10y)	-	-	-	32	16	8	16

		'07	'08	'11	'12			'13	'15
		6	7	12	4	5	6	9	4
C	263 (♀, 14y)	-	-	-	-	8	4	2	2

a) Year, b) Month, c) Inside of parenthesis shows gender and age (years old), d) -: <2.

to those of systemically infecting herpesvirus in horses [8, 11, 16]. Another possibility is that oral administration of valacyclovir suppressed the increase in antibody titers [31]. The *Bam*HI DNA profile of YS-1 was almost the same of that of the prototype of EHV-3 [30]. Similar *Bam*HI DNA profiles of SS-1 and Iwate-1 were observed in field EHV-3 isolates in Australia and the U.S.A. [9, 19]. The remarkable size difference observed between *Bam*HI-A fragments of YS-1 and SS-1 was due to the lack of two *Bam*HI restriction sites in YS-1, each positioned at 958 bp from both ends of the *Bam*HI-A fragment of YS-1. Sequence analyses of gG, gB, gC and VP13/14 revealed that SS-1, YS-1 and Iwate-1 were slightly different. These results suggested that there might be several types of EHV-3 circulating in the horse population in Japan.

Barrandeguy [2] reported that there were at least four genetically distinguishable EHV-3 strains based on the partial sequence of the gG gene obtained from 25 field isolates. Three base substitutions in the gG gene have been found at positions 904, 1,103 and 1,264, which result in strains CAT (Australia), AAT (the U.S.A. and Brazil), CAG (Argentina) and ACT (Argentina). SS-1 belongs to the CAG type, and YS-1 and Iwate-1 belong to the ACT type. Therefore, in these criteria, Japanese EHV-3 isolates belong to the same group that Argentina isolates belong to, but are different from isolates of the U.S.A., Brazil and Australia.

The sources of EHV-3 infection in the two stallions were unclear. In both stallion farms, the female progenital skin and mucosa were carefully observed before mating [31]. One possible reason was that the horse initiating the infection might not have shown clinical signs, and careful examination might have revealed one or few ulcerative lesions inside the progenital mucosa from which reactivated virus was shed and transmitted to the mating partner [29]. Epidemiological data suggested that the original viral source of an outbreak of ECE might be either a visiting mare brought onto the stud farm for breeding or virus reactivated from a member of the resident stallion or mare population [8, 12, 15, 26].

The sero-epidemiological study revealed that the EHV-3 positive rate was 4.1% and that EHV-3 infection might be widespread in the horse population in Japan. In Argentina, almost 50% of adult, breeding-age mares were seropositive and presumably latently infected [5]. In Mongolia, EHV-3 antibody-positive horses were detected in most provinces, and the positive rate (22.9%) was higher than that in our study [24]. One of the reasons for this high rate might be that horse reproduction in Mongolia occurred

through natural mating and the rate of artificial insemination was low.

Our study showed that the infection rate of stallions was higher than that of broodmares. This suggested that infection risk for stallions might be higher than that for broodmares due to mating with many broodmares. In the retrospective study, all of the four EHV-3 antibody-positive horses showed sero-conversion in the breeding season. One stallion (No. 411) showed sero-conversion against EHV-3 twice without any clinical symptoms of ECE. The antibody-positive period after the first sero-conversion was only two months, but that after the second sero-conversion was at least one year, suggesting that re-infection or reactivation of EHV-3 might have occurred. Furthermore, although two other stallions (Nos. 825 and 833) in Farm A and one broodmare (No. 263) in Farm C showed sero-conversion against EHV-3 during the rearing period in each farm, no clinical case of ECE was observed. These results suggested that asymptomatic infection might have occurred frequently. In Japan, it was reported that sero-conversion against EHV-3 was observed despite no development of progenital regions in stallions and broodmares [26]. Without mating with a stallion, the antibody titer against EHV-3 increased in some antibody-positive broodmares [27]. These increases of the antibody titer might be due to reactivation of EHV-3 like other latent herpesviruses [1, 22]. Barrandeguy *et al.* [5, 7] reported that latently infected mares may not only reactivate the virus and shed it but also be re-infected and shed a second virus to other horses. Viral excretion without clinical signs can therefore occur and contribute to the venereal and mechanical transmission of EHV-3.

In conclusion, EHV-3 was firstly isolated from ECE of two stallions reared in different farms in Japan. The results of sero-epidemiological survey suggested that EHV-3 infection might exist widely without clinical symptoms in Japanese horses and that infection might occur mainly in the breeding season in Japan.

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