FULL PAPER *Virology*

Occurrence of Equine Coital Exanthema in Pastured Draft Horses and Isolation of Equine Herpesvirus 3 from Progenital Lesions

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ABSTRACT. During the period from 2001 to the following year, progenital diseases had been epidemic among the draft stallions and mares pastured together in Iwate Prefecture, the northeastern district of Japan. A stallion and 8 of 31 mares were affected in 2001, and 1 of 2 stallions and 10 of 36 mares in 2002. The clinical symptoms consisted of the formation of papules, pustules, ulcers and scabs on the progenital skin and mucosa in stallions and mares. In 2002, Equine herpesvirus 3 (EHV3) was isolated from 2 mares and the glyco protein G gene of the virus detected from a stallion and 4 mares by polymerase chain reaction. Serum neutralizing tests showed that 12 of 38 horses, 10 clinically and 2 subclinically affected, changed to be positive for the EHV3 antibody. The results suggest that the horses were affected with equine coital exanthema (ECE) through coitus. Five mares with the antibody at the pre-pastured period may have been the possible origins of EHV3 infection in 2002, although the exact origin in 2001 remains unknown. The artificial insemination was performed for the prevention of ECE spreading through coitus on the pasture in 2003. There was no epidemic of the disease in 31 mares, although 3 mares with the antibody at the pre-pastured period showed the significant increase in the titers during the pastured period.

KEY WORDS: coital transmission, equine coital exanthema, equine herpesvirus 3, isolation, seroepidemiology.

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Equine coital exanthema (ECE) caused by equine herpesvirus 3 (EHV3) is an acute, venereal disease characterized by the formation of papules, vesicles, pustules and ulcers on the vaginal and vestibular mucosa as well as on the skin of the penis, prepuce and perineal region [2–4, 8, 11, 14, 15, 17–20]. In general, the infections are subclinical or mild [20]. There are no systemic symptoms followed by complete healing within 14 days later when uncomplicated [19, 20]. EHV3 is primarily transmitted through coitus [1, 13, 18–20], although there are also evidences supporting the possibility of non-coital spreading through infected fomites and contacts except for coitus [3, 4, 11, 13, 15, 19, 20].

Abortion and infertility are not associated with ECE [14, 19, 20]. Therefore, the management of the disease usually consists of symptomatic treatment and temporal removal of the affected horses from the herd [20]. Latency of EHV3 has not been formerly demonstrated in field cases, but several outbreaks may be initiated by reactivation of the latent virus in association with coitus [20] and recurrent diseases also occur [2].

EHV3 has been isolated in many countries such as the United States [3, 4], Australia [6, 17, 18], Canada [8], Denmark [2], Norway [11] and England [7]. In Japan, there are no reports on the isolation of EHV3 from affected horses as far as we know, although the presence of horses with clinical symptoms similar to those of ECE had been occasionally noticed in limited regions. The purpose of this paper is to describe the virological findings of the pastured horses affected with ECE.

MATERIALS AND METHODS

Physical and virological examinations had been performed on the horses pastured during the period from 2001 to 2003. One stallion (A) and 31 mares were examined in 2001, 2 stallions (B, C) and 36 mares (Nos. 1–36) in 2002, and 31 mares (Nos. 37–67) in 2003.

Physical examination: The female progenital skin and mucosa were carefully observed on the following dates: 13 May when began to be pastured, 27 and 30 May, 14 June, 12 July, 23 August, 20 September and 18 November when left the pasture in 2001; 12 May, 12 and 27 June, 8 July, 2 August, 26 September and 23 November in 2002; 11 and 27 May, 12 and 25 June, 17 July, 21 August, 18 September and 16 November in 2003. The penis, prepuce and scrotum of a stallion (A) were examined on 24 May 2001, and 2 stallions (B, C) on 12 June and 4 July 2002.

Samples: For the detection of EHV3, 4 swabs and 3 biopsy tissues were obtained from the lesions of a stallion (C) and 4 mares (Nos.1, 2, 5, 6) on 8 July 2002. Swabs were placed in 0.5 m*l* of phosphate-buffered saline (PBS). Tissue specimens were emulsified and suspended 10% (w/v) solution in PBS. Each sample was centrifuged to pellet debris and the supernatant fluid was kept at –80°C prior to use.

The paired sera were collected from 2 stallions (B, C) and 36 mares (Nos. 1–36) on 8 July and 2 August 2002. Serum samples of those and one other stallion (A) were also collected in mid-February 2002. In 2003, 112 sera were obtained from 31 mares (Nos. 37–67) on 12 June, 17 July and 18 September as well as in mid-February. Samples

were inactivated at 56°C for 30 min and stored at –20°C until use.

Cell culture and virus: Equine kidney (EK) and testis (ET) cells were cultured in 96-well, flat-bottomed microplates for the virological examination, because EHV3 is highly host specific and grows only in cells of equine origin [3, 18, 20]. The culture medium consisted of Eagle's minimum essential medium supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL, U.S.A.), 0.3% tryptose phosphate broth, 0.03% L-glutamin and 0.15% sodium bicarbonate. The 1118 strain [3] of EHV3 was propagated in EK cells and used as the reference one in this study. This virus was originally isolated by Dr. J. T. Bryans (Kentucky University) and maintained in National Institute of Animal Health (Tsukuba, Japan).

Virus isolation: Fifty μl of each supernatant fluid was inoculated into each of 3 well cultures of EK cells from which the medium had been removed. After adsorption at 37°C for 60 min, the cultures were washed twice with the medium, received the fresh medium and incubated at 37°C. When no cytopathic effects (CPE) appeared on the cells for 7 days, 3 blind passages were performed.

Polymerase chain reaction (PCR) for detection of EHV3 gene and DNA sequencing: The molecular techniques were used for the identification and sensitive detection of EHV3. Virus DNA was extracted from isolates having been cloned 3 times with the limiting dilution method, field samples and the 1118 strain using a QIAamp DNA Mini Kit (QIAGEN K. K., Japan) according to the manufacturer's instruction.

A primer pair capable of amplifying the partial-length glycoprotein G (gG) gene of EHV3 was prepared as described by Dynon *et al.* [5]. The sequences (5'–3') of oligonucleotides and their positions in the genome of EHV3 334/74 strain are as follows: GCGCTCTCTCGGCCTTGCCAG (sense: 792–812); GGCGTCTCGAAAAGCGAGAG (antisense: 1309–1290) [5, 10]. Amplification reactions were performed in a 20 μl reaction mixture containing 2.5 mM of $MgCl_2$, 0.2 mM of dNTP, 0.5 μM of each primer, 0.5 unit of the Taq polymerase (Applied Biosystems, Japan) and 2 μl of template.

Cycling was carried out using Takara PCR Thermal Cycler. Following an initial denaturing step at 95°C for 10 min, 40 amplification cycles were conducted consisting of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min [5]. The reaction was completed by a final extension step at 72°C for 10 min. The amplified products were analyzed by electrophoresis in a 2% agarose gel and detected by staining with ethidium bromide.

The PCR amplicons of isolates and the 1118 strain were purified with a Microcon-PCR (Millipore, U.S.A.) and directly sequenced in both directions with the same primers used to generate the PCR products. Sequencing was done in an ABI PRISM 377 based on the incorporation of fluorescein-labelled dideoxynucleotide terminators. The determined nucleotide sequences were aligned using the multiple alignment programs from the DNASTAR software package. The sequences of gG gene of the 334/74 strain [10] were taken from GenBank (Accession number AF081188).

Serum neutralizing (SN) test: EHV3 shows no serologic cross-reactivity with other equine herpesviruses by neutralizing tests [9, 19, 20]. Neutralizing test of sera obtained in 2002 or 2003 was carried out using EK or ET cells. Briefly, serial twofold dilutions, beginning with a 1:2 dilution, of the serum were mixed with an equal amount of the 1118 strain containing a 200 median tissue culture infectious dose per 0.1 m*l* before incubation at 37°C for 60 min. One well of cell cultures was inoculated with 0.1 m*l* of each virus-sample mixture and incubated at 37°C. The results were read 7 days postinoculation. SN titer was expressed as the reciprocal of the highest dilution that completely inhibited CPE.

RESULTS

Occurrence: One 7-year-old stallion (A) mating with 31 draft mares developed the progenital lesions as described below on the pasture located in Iwate Prefecture, the northeastern district of Japan, on 24 May 2001. The lesions consisted of extensive ulcerations over the entire shaft of the penis and numerous papules, pustules and scabs on the prepuce and scrotum (Fig. 1). Similar lesions were observed on the vestibular, vulval and perineal skin and mucosa in the 8 mares 3 to 6 days later. Such symptoms, from which ECE is suspected as a causal disease, had never been found in any stallions and mares on this pasture in 2000 or earlier. Any affected horses showed no febrile response. Those were segregated from unaffected horses. The lesions were treated with antibiotics and disinfectants and followed by full healing within 3 weeks later. Two mares (Nos. 32, 33) introduced from the other prefecture in September 2000 had been pastured with other horses since the following spring.

From 12 May to 23 November in 2002, 36 mares (Nos. 1-36) ranging 2–15 years of age had been pastured after verification of no progenital lesions by the physical examination. Twenty- six (Nos. 1–9, 11–22, 32–36) of 36 mares had been grazed on the pasture in the previous year too, with development of the progenital lesions in 3 mares (Nos. 32–34). One stallion (B) aged 9 years had been pastured for the breeding from 20 May to 16 June and another stallion (C) aged 11 years from 23 June to 4 July. On 4 and 8 July, a stallion (C) and 10 mares (Nos. 1–3, 5–10, 32) developed the progenital lesions similar to those observed in the previous year.

In 2003, 31 mares (Nos. 37–67) had been pastured without any stallions from 11 May to 16 November. Artificial insemination (AI) was performed on 29 of 31 mares on the pasture. The semina were obtained from the EHV3-free stallion and the instruments for AI were carefully disinfected prior to use. The conception was confirmed in 24 mares using an ultrasonography in June or July. No progenital lesions had been found in any horses during the pastured period.

Virus detection: Isolation and PCR for detection of EHV3 from the lesions were performed, because ECE was sus-

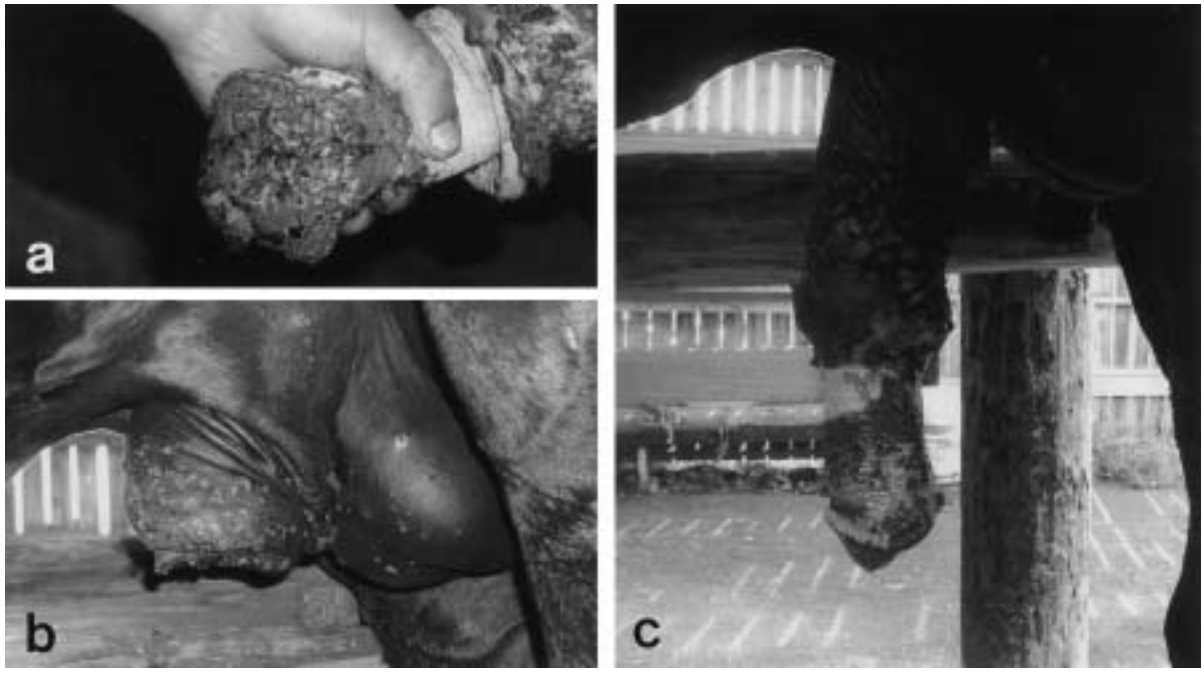

Fig. 1. Progenital lesions of two stallions. a: Ulcers and scabs on the penis and prepuce of stallion A. b: Numerous papules, pustules and scabs on the prepuce and scrotum of stallion C. c: Extensive ulcerations on the penis of stallion C.

pected from the clinical symptoms observed in affected horses. Cytopathic agents were isolated from 2 (Nos. 1, 2) of 5 horses at the first passage level, 12 hr to 3 days postinoculation. CPE were characterized by cell rounded and syncytial formation (Fig. 2). By PCR amplifying the partial-length gG gene of EHV3, the identical products with the 1118 strain in size were detected from both isolates (Fig. 3). The nucleotide sequences of fragments obtained from both isolates were identical and 518 bp in size. The nucleotide homology of isolates' gG gene was 99.4% compared with each of the 1118 or 334/74 strains (Fig. 4). On the basis of these results, the isolates from 2 mares (Nos. 1, 2) were identified as EHV3 and designated the Iwate-1 and -2 strains, respectively. The identical PCR products with isolates in size were also detected from all field materials of 5 horses (C, Nos. 1, 2, 5, 6) (Table 1 and Fig. 3).

Serological epidemiology: The antibody was detected from the stallion (A) at the post-pastured period in 2001. Two stallions (B, C) and 10 mares (Nos. 1–10) changed to be positive for the antibody during the pastured period in 2002, although 2 horses (B, No. 4) of those developed no progenital lesions. The significant (4-fold or more) increase in the antibody titers was found in 4 mares (Nos. 1–4) between 8 July and 2 August. While, a mare (No. 5) showed a decrease in the titer and 5 (Nos. 6–10) kept the same titer level on 2 August following the positive conversion on 8 July. Twenty- one mares (Nos. 11–31) remained negative. The other 5 mares (Nos. 32–36), including 2 mares (Nos. 32, 33) introduced from the other prefecture, had possessed the antibody since the pre-pastured period in 2002, with the significant increase of the titer in 2 mares (Nos. 34, 36) and the decrease in a mare (No. 33) during the pastured period (Table 2).

Fig. 2. CPE at 12 hr postinoculation induced by the progenital swab of affected mare No. 2 on the first passage in EK cells. Note cell rounded and syncytial formation.

There were no mares that changed to be positive for the antibody during the pastured period in 2003, when 20 negative (Nos. 37–56) and 11 positive (Nos. 57–67) mares for the antibody at the pre-pastured period had been pastured together. Three (Nos. 57–59) of 11 positive mares showed the significant increase in the antibody titers between June and July. A decrease of the titers to an undetectable level

was found in 2 mares (Nos. 57, 60), followed by the subsequent increase in a mare (No. 57) (Table 3).

DISCUSSION

Progenital diseases had been epidemic among the stallions and mares pastured together during the period from 2001 to 2002. Affected stallions and mares developed several clinical symptoms closely similar to those [2, 8, 11, 14, 17–20] of ECE previously reported. One stallion (A) having developed the symptoms in 2001 possessed the EHV3 antibody at the post-pastured period. EHV3 was isolated from the lesions of 2 mares (Nos. 1, 2) and gG gene of the same virus detected from 5 horses (C, Nos. 1, 2, 5, 6) in 2002. Two stallions (B, C) and 10 mares (Nos. 1–10) changed to be positive for the antibody during the pastured period in 2002. The facts suggest that the horses were affected with ECE and that EHV3 was epidemic through coitus as one of important means of the transmission. Five mares (Nos. 32–36) with the antibody at the pre-pastured period may have been the possible origins of EHV3 infection in 2002, although the exact origin in 2001 remains unknown.

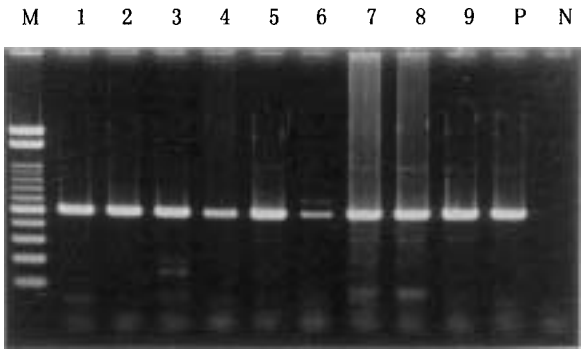

Fig. 3. PCR for detection of gG gene of EHV3 from isolates in EK cells and field samples. Lanes 1 and 2: Isolates from tissue of mare No. 1 and swab of No. 2. 3–6: Swabs of stallion C, Nos. 1, 2 and 6. 7–9: Tissues of C, Nos. 1 and 5. P: EK cells supernatant infected with EHV3 1118 strain. N: Uninfected EK cells supernatant. M: Molecular weight marker (100 bp DNA Ladder, TOYOBO Co., Ltd.).

On the experimental infection with EHV3, the serum antibody titers developed to a maximum level between 3 and 4 weeks later and subsequently declined slowly [16]. Following one stallion (B) left the pasture on 16 June, another stallion (C) had been grazed until 4 July, and ECE symptoms in 10 mares (Nos. 1–3, 5–10, 32) were observed on 8 July in 2002. The antibody titers increased significantly in 4 mares (Nos. 1–4) and decreased in a mare (No.5) on 2 August following the positive conversion on 8 July. The results suggest that the former mares infected with EHV3 later than the latter and that both stallions were possibly related to ECE spreading in 2002.

Spontaneous reactivation of latent herpesviruses is associated with various stresses and followed by an increase in the antibody titers [12]. In 2003 when AI was performed for the prevention of ECE spreading through coitus, there were no mares that developed ECE symptoms or changed to be positive for the antibody. Two (Nos. 34, 36) and 3 (Nos. 57–59) mares with the antibody at the pre-pastured periods in 2002 and 2003 showed the significant increase in the titers during the pastured periods. The increase may have

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Isolate-1   792  GCGCTCTCTCGGCCTTGCCAGGGGCCCTCAATCCCTGCTCATTGGCGCCCTGGGACTTCGCATCCTGAGCCAGCGCTGGTACATGCTGCCGGGCGAGACGTACGACCAGC  901
Isolate-2   792  ..............................................................................................................  901
1118        792  ..............................................................................A...............  901
334/74      792  ..............................................................................A...............  901

Isolate-1   902  TGAGGCAAAATTCCAGAGGGTCTGCCCGCGGCGCAGACAGGGAATCCGCGGCCGACGTAACAGAACCCGAAGAAAAGCCTTCGGAAAAAACCCCCGCTTCTCCCACCGAT  1011
Isolate-2   902  ..............................................................................................................  1011
1118        902  ..............................................................................-...............  1010
334/74      902  ..C...........................................................................................................  1011

Isolate-1  1012  GACGAGGAGAAAGAAGAAGAGGAAAACGGGGATAACGAGCCAACCCCAGCGCCACCGGCCCCGGGATGCCACGAGCAAGACGCACCTGCCGCCGGAGACGGATCTCCGTG  1121
Isolate-2  1012  ..............................................................................................................  1121
1118       1011  ..............................................................................A...............  1120
334/74     1012  ..............................................................................A...............  1121

Isolate-1  1122  GTACACCGGCGGCATTCTCGTGTCCGGTCCTGAGTGCGGACAGCAGAAGGGGACCAACTACGCGGGTATCGGCTTTCTCATCTTGGGAGTGTGTCTCCTCATCGGCCTCA  1231
Isolate-2  1122  ..............................................................................................................  1231
1118       1121  ..............................................................................................................  1230
334/74     1122  ..............................................................................................................  1231

Isolate-1  1232  TTGTCTACGTTTGCGTCCTGCGGTCCAGAGTCTCCGAGCGCAAGCTCCACAACAGCTACTCTCGCTTTTCGAGACGCC  1309
Isolate-2  1232  ..............................................................................  1309
1118       1231  ..............................................................................  1308
334/74     1232  ..............................................................................  1309
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Fig. 4. Comparison of the nucleotide sequences of gG gene from 2 field strains (Isolates-1 and -2 recovered from mare Nos. 1 and 2) with those of EHV3 1118 and 334/74 strains. The identical nucleotide is indicated by a dot and the gap shown by a dash.

Table 1. Detection of EHV3 by isolation or PCR from progenital lesions of 5 horses pastured in 2002

Horse[a)]	Examined materials	Virus isolation	PCR
C	Swab	–	+
	Tissue	–	+
1	Swab	–	+
	Tissue	+	+
2	Swab	+	+
5	Tissue	–	+
6	Swab	–	+

a) The alphabet indicates a stallion and the numbers mares.

Table 2. ECE symptoms and serum EHV3 neutralizing titers in pastured horses

Horse[a)]	2001	2002			
	ECE symptoms	ECE symptoms	Pre-pastured (February)	Post-pastured (July)	Post-pastured (August)
A	+	NT	64≤	NT	NT
B	NT	–	<2	32	64≤
C	NT	+	<2	16	16
1	–	+	<2	<2	4
2	–	+	<2	<2	64≤
3	–	+	<2	4	16
4	–	–	<2	2	16
5	–	+	<2	8	2
6	–	+	<2	8	4
7	–	+	<2	4	4
8	–	+	<2	4	4
9	–	+	<2	2	4
10	NT	+	<2	2	2
11–22	–	–	<2	<2	<2
23–31	NT	–	<2	<2	<2
32	+	+	4	8	8
33	+	–	64≤	8	8
34	+	–	4	16	8
35	–	–	8	4	4
36	–	–	2	16	16

a) The alphabets indicate stallions and the numbers mares.
NT: Neither pastured nor tested.

Table 3. Serum EHV3 neutralizing titers in horses pastured in 2003

Horse	Pre-pastured (February)	Post-pastured (June)	Post-pastured (July)	Post-pastured (September)
37–51	<2	<2	<2	<2
52–54	NT	<2	<2	<2
55	<2	<2	NT	<2
56	NT (<2)[a)]	NT	NT	<2
57	2	<2	4	8
58	2	2	8	8
59	NT (4)	4	16	16
60	4	NT	2	<2
61	8	8	8	4
62	8	16	16	8
63	8	8	4	8
64	4	2	4	4
65	8	8	8	8
66	NT (4)	8	16	16
67	NT (4)	NT	16	8

a) The values in parenthesis indicate serum neutralizing titers in August 2002.
NT: Not tested.

resulted from reactivation of the latent EHV3. It seems likely that a horse-to-horse transmission except for coitus would be unusual on the pasture and that the reactivated virus spread in the pastured herd when the natural breeding was applied.

Two horses (B, No. 4) may have been subclinically affected with ECE since both ones changed to be positive for the antibody without developing any symptoms in 2002. A decrease to an undetectable level followed by a subsequent increase of the antibody titers was found in a mare (No. 57) in 2003. The results may suggest that SN tests should be performed more than twice to find out potential carriers of EHV3 such as subclinically affected or pretended seronegative horses.

Further surveys will be conducted on the factors associated with reactivation of the latent EHV3 in the pastured horses.

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