Genomic Diversity among Equine Herpesvirus-4 Field Isolates

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ABSTRACT. Infection with equine herpesvirus-4 (EHV-4) is a major cause of respiratory tract disease, equine rhinopneumonitis, in horses. Although the full sequence of EHV-4 has been reported, genomic differences among EHV-4 field isolates have not yet been characterized. In this study, the genomic diversity between 23 Japanese EHV-4 isolates was analyzed by digestion with restriction endonucleases (*Bam*HI, *Bgl*II, *Eco*RI, *Sac*I, and *Sal*I) and polymerase chain reaction (PCR). The restriction endonuclease digestion patterns of the EHV-4 field isolates showed distinct differences which included mobility shifts of some fragments as well as loss and/or gain of fragments. Two EHV-4 genes containing repeat sequences, ORFs 24 and 71, were amplified by PCR and the amplified fragments were compared among the field isolates. The sizes of the amplified fragments varied among epizootiologically unrelated isolates, while the fragments of related isolates had the same size. The observed genomic diversity among EHV-4 field isolates may be a useful tool for epidemiological study of equine rhinopneumonitis by EHV-4 infection.
KEY WORDS: epizootiology, equine herpesvirus-4, field isolates, genomic diversity.

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Equine herpesvirus-1 (EHV-1) and -4 (EHV-4) are the major causative agents of equine respiratory disease in horse, also known as equine rhinopneumonitis. In Japan, EHV-1 is responsible for causing respiratory disease, particularly in racehorses in the winter season, and may also cause occasional neurological disorders and abortion among mares. In contrast, EHV-4 causes respiratory disease throughout the year among all horse populations, including racehorses, foals, yearlings, rearing horses and dams [14]. As a result, these viral infections have had a negative economical impact on the Japanese horse industry. EHV-1 and -4 are closely related both genetically and antigenically, and are cross-reactive in virus-neutralization tests, complement-fixation tests, and immunoblot analysis. Recently, a type-specific ELISA was developed and applied for serological diagnosis and sero-epizootiological study of both virus infections [6, 12, 13, 15, 31, 32].

The complete genome sequence of EHV-1 was reported in 1992 [27], and the genomic diversity among EHV-1 field isolates has been well characterized using restriction endonuclease analysis [1, 2, 4, 5, 10, 16, 18, 19, 22–26, 29]. In addition, the polymerase chain reaction (PCR) has been applied for the diagnosis and epizootiology of EHV-1 infection, and for discrimination between EHV-1 and -4 infections [3, 9, 17, 20, 30].

Recently, the full genomic sequence of EHV-4 NS80567 strain was reported [28]; however, only a few reports have studied the genomic diversity of EHV-4 field isolates [2, 7, 24]. We analyzed the genomic variability among EHV-4 field isolates by restriction endonuclease digestion and PCR, and identified unique markers for each isolate.

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MATERIALS AND METHODS

Viruses and cells: The EHV-4 field isolates are listed in Table 1. The H45 strain was isolated from an aborted fetus in Japan and has been used as the Japanese prototype strain of EHV-4 [21]. The TH20 strain was isolated from a colt suffering from respiratory disease and has been used as a standard EHV-4 strain [8]. The other 21 strains were isolated between 1981 and 1991 and propagated in fetal horse kidney (FHK) cells (Table 1). Field isolates were used within the third passage and were never plaque-purified. Two pairs of isolates, 88c160 and 88c162, as well as 88c180 and 88c186, were simultaneously isolated from different horses from the same farm and are therefore considered to be epizootiologically related. Since the other viruses were isolated from different farms or at different periods, these were tentatively judged to be epizootiologically unrelated.

DNA extraction: Twenty-one field isolates and the TH20 and H45 strains were used for DNA analyses. Viruses were propagated at low multiplicities of infection in FHK cells. Infected cells were treated with l% sodium dodecyl sulfate and proteinase K (0.1 mg/ml) in 0.1 M Tris-HCl, 0.1 M NaCl$_2$, 5 mM EDTA, pH 9.0 at 37°C overnight. Following DNA extraction with phenol and chloroform-isoamylalcohol (24:1), ethanol-precipitated DNAs were dissolved in water.

Digestion with restriction endonuclease: Viral DNAs were digested with *Bam*HI, *Bgl*II, *Eco*RI, *Sac*I, and *Sal*I. Digested samples were separated by electrophoresis on a 0.7% agarose gel for 18.5 hr at 20V. Gels were stained with ethidium bromide and analyzed.

Amplification by PCR: PCR primers are listed in Table 2. The complete open reading frames (ORFs) of EHV-4 gB, gC, gD and gp2 were amplified from viral DNA by PCR and

Table 1.   EHV-4 strains and field isolates used in this study

Strains and field isolates	Prefectures	Isolation site	Age	Disease	Year of isolation
H45	Hokkaido	Aborted fetus	–	Abortion	1959
TH20	Aomori	Nasal swabs	0	Respiratory disease	1962
81c123	Miyazaki	Nasal swabs	1	Respiratory disease	Jul/1981
82c53	Ibaraki	plasma	3	Respiratory disease	Mar/1982
82c72	Ibaraki	Nasal swabs	3	Respiratory disease	Mar/1982
82c208	Ibaraki	Nasal swabs	2	Respiratory disease	Dec/1982
82c230	Shiga	plasma	2	Respiratory disease	Dec/1982
85c2	Tochigi	Nasal swabs	1	Respiratory disease	Mar/1985
86c1	Hokkaido	Nasal swabs	2	Respiratory disease	May/1986
87c16	Hokkaido	Nasal swabs	0	Respiratory disease	Jul/1987
87c18	Hokkaido	Nasal swabs	1	Respiratory disease	Jul/1987
87c34	Tochigi	plasma	1	Respiratory disease	Dec/1987
88c61	Hokkaido	Nasal swabs	1	Respiratory disease	Apr/1988
88c68	Tochigi	plasma	2	Respiratory disease	Dec/1988
88c159	Hokkaido	Nasal swabs	0	Respiratory disease	Sep/1988
88c160[a]	Hokkaido	Nasal swabs	0	Respiratory disease	Oct/1988
88c162[a]	Hokkaido	Nasal swabs	0	Respiratory disease	Oct/1988
88c176	Hokkaido	Nasal swabs	0	Respiratory disease	Nov/1988
88c178	Hokkaido	Nasal swabs	0	Respiratory disease	Dec/1988
88c180[b]	Hokkaido	Nasal swabs	0	Respiratory disease	Dec/1988
88c186[b]	Hokkaido	Nasal swabs	Dam	No clinical sign	Dec/1988
88c190	Hokkaido	Nasal swabs	1	Respiratory disease	Oct/1988
91c1	Saga	Aborted fetus	–	Abortion	Mar/1991

a) Epidemiologically related isolates.
b) Epidemiologically related isolates.

Table 2.   Primers and condition of PCR to compare genomic diversity among EHV-4 stains and field isolates

Primer no.	Sequence(5'⇒3')	Nucleotide position[a]	PCR (30 cycles)		
			Denaturing	Annealing	Extension
gB4-F	GGGAATTCATGTCCACTTGTTGCCG	61147–61163	92°C 1'	49°C 1'	72°C 3'
gB4-R	TTCTCGAGTTAAACCATTTTTTCGC	64074–64058			
gC4-F	GGGAATTCATGGGTTTGGTAAATAT	22396–22380	92°C 1'	49°C 1'	72°C 2'
gC4-R	TTCTCGAGTTAGAAGTCTGCTTTCT	20939–20955			
gD4-F	TTGGATCCGTATGTCTACCTTCAAG	129796–129812	92°C 1'	53°C 1'	72°C 2'
gD4-R	TTCTCGAGTTACGGAAGCTGGGTAT	131006–130990			
VLTP4-F	AAGCACGGCGGTTGGGTCTA	43919–43938	92°C 1'	57°C 1'	72°C 2'
VLTP4-R	GTATTCTCCCGGTTCCTGTG	45444–45425			
gp2-4-F	AGCGATGGGGTTCATCTA	127451–127468	92°C 1'	54°C 1'	72°C 3'
gp2-4-R	TTAATATACGGACGCCCG	129707–129690			

a) Based on the genome of EHV-4 NS80567 strain (Accession No. AF030027).

the *C*-terminal region of very large tegument protein (VLTP) was also amplified by PCR using primers VLTP4-F and VLTP4-R.   PCR using viral DNAs as templates was performed by Takara LA PCR kit (Takara, Japan) at the conditions as described in Table 2.   Amplified samples were separated by electrophoresis on 1–2% agarose gels.   Gels were stained with ethidium bromide and analyzed.

RESULTS

Comparison of restriction endonuclease digestion patterns among EHV-4 strains and field isolates: The genomic DNA of EHV-4 from 20 horses with respiratory tract disease, two aborted fetuses, and one dam with no clinical symptoms whose foal suffered from respiratory tract disease were analyzed by digestion with restriction endonucleases *Bam*HI, *Bgl*II, *Eco*RI, *Sal*I, and *Sac*I.   The results showed mobility shifts of some fragments, specifically H (*Bam*HI),
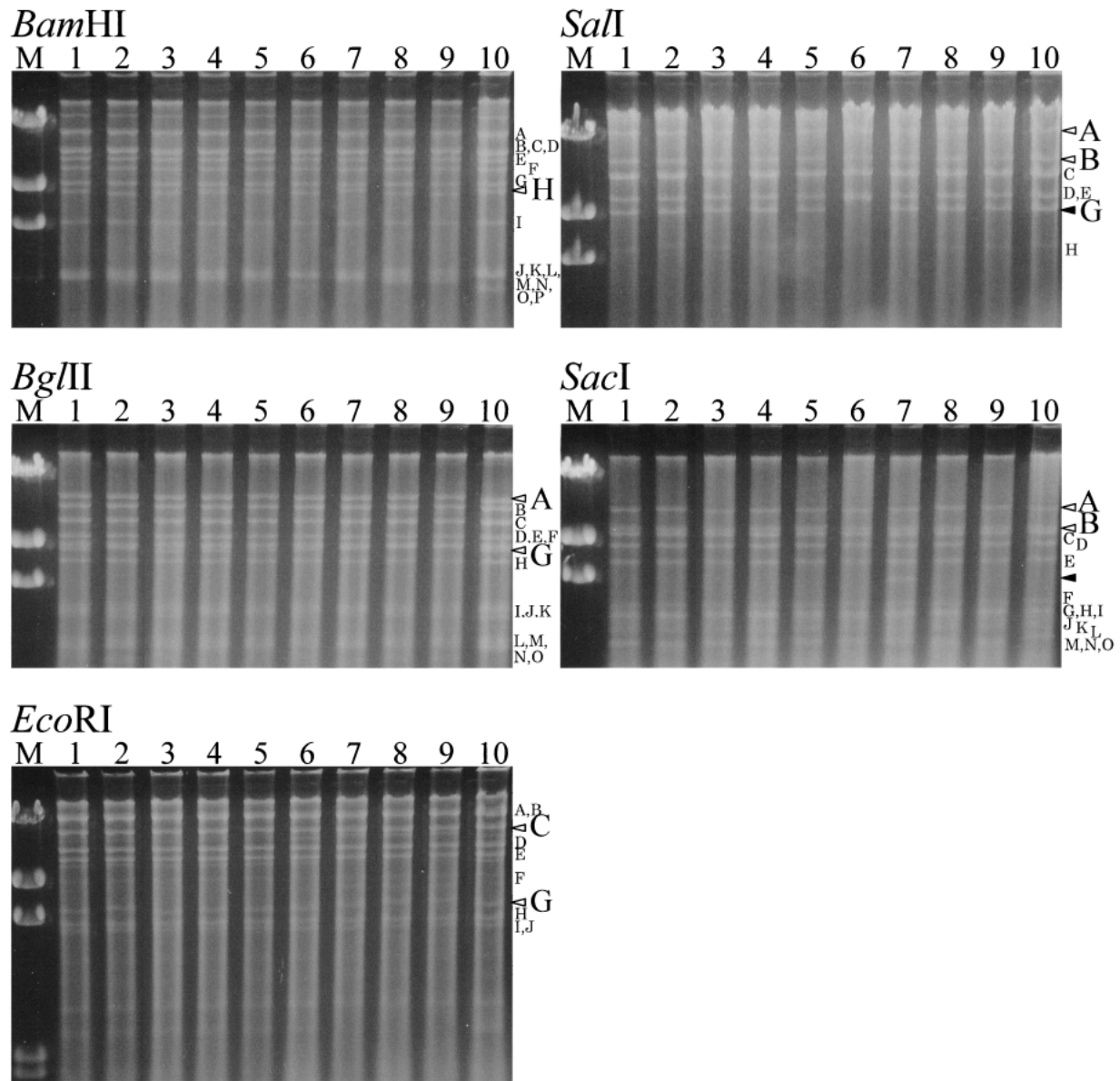
Fig. 1.   Representative results of digestion with five restriction rendonucleases, *Bam*HI, *Bgl*II, *Eco*RI, *Sal*I and *Sac*I. Lane 1; 88c16, lane 2; 88c68, lane 3; 88c159, lane 4; 88c160, lane 5; 88c162, lane 6; 88c176, lane 7; 88c178, lane 8; 88c180, lane 9; 88c186 and lane 10; 91c1. Open arrowheads show the fragments with mobility shifts among field isolates. Bold arrowheads show the fragments with loss or gain among field isolates. Lane M means a marker for molecular weights, *Hin*dIII-digested lamda phage DNA.

A and G (*Bgl*II), C and G (*Eco*RI), A and B (*Sal*I) and A and B (*Sac*I) (Figs.1 and 2). In addition, loss and/or gain of fragments was observed in some isolates. In the H45 strain, the 18.5-kbp C (*Eco*RI) and 3.3-kbp K (*Eco*RI) fragments observed in TH20 disappeared and a new, approximately 22-kbp, fragment was detected. In H45 and 88c176, the 9.3kbp-G (*Sal*I) fragment disappeared and a new, approximately 10 kbp, fragment was detected. *Sac*I digestion of H45, 81c123, 82c72, 82c230, and 88c178, resulted in a new, approximately 6-kbp, fragment. In 88c190, an approximately 9-kbp *Sac*I fragment was detected (data not shown).

In summary, these results indicate that 23 field isolates or laboratory strains were divided into at least five genotypes by restriction endonuclease digestion pattern, designated as genotypes A to E. We expect that some fragments with different mobility were not identified because of other fragments with similar molecular weights. In addition, fragments with small molecular weights (less than approximately 4 kbp) were not analyzed in this study, because those were difficult to detect.

Identification of regions with mobility shifts among EHV-4 field isolates: The sites recognized by the restriction endo-
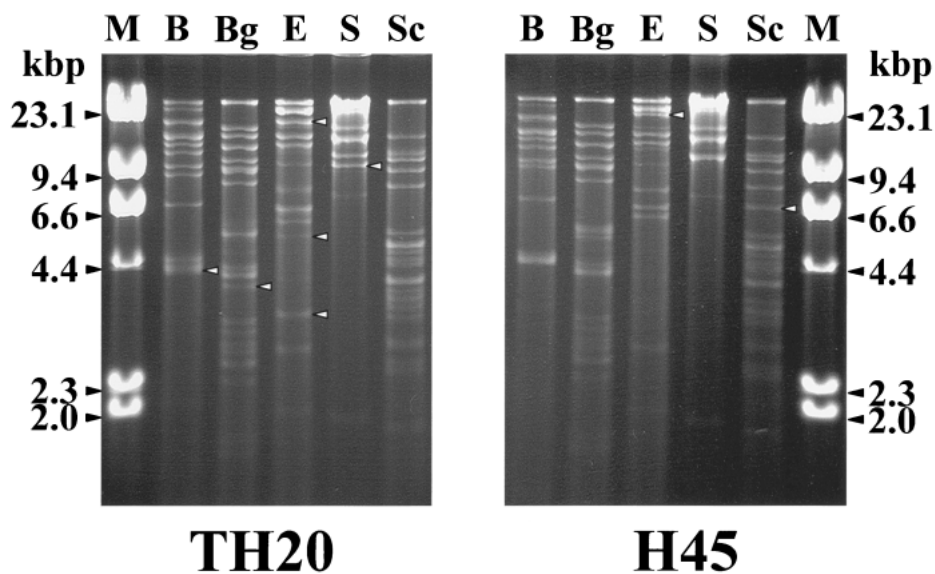
Fig. 2.   Comparison between Japanese prototype strains, TH20 and H45 by restriction endonuclease digestions. Open arrowheads show fragments with remarkable changes. B; *Bam*HI, Bg; *Bgl*II, E; *Eco*RI, S; *Sal*I and Sc; *Sac*I. Lane M means a marker for molecular weights, *Hin*dIII-digested lamda phage DNA.
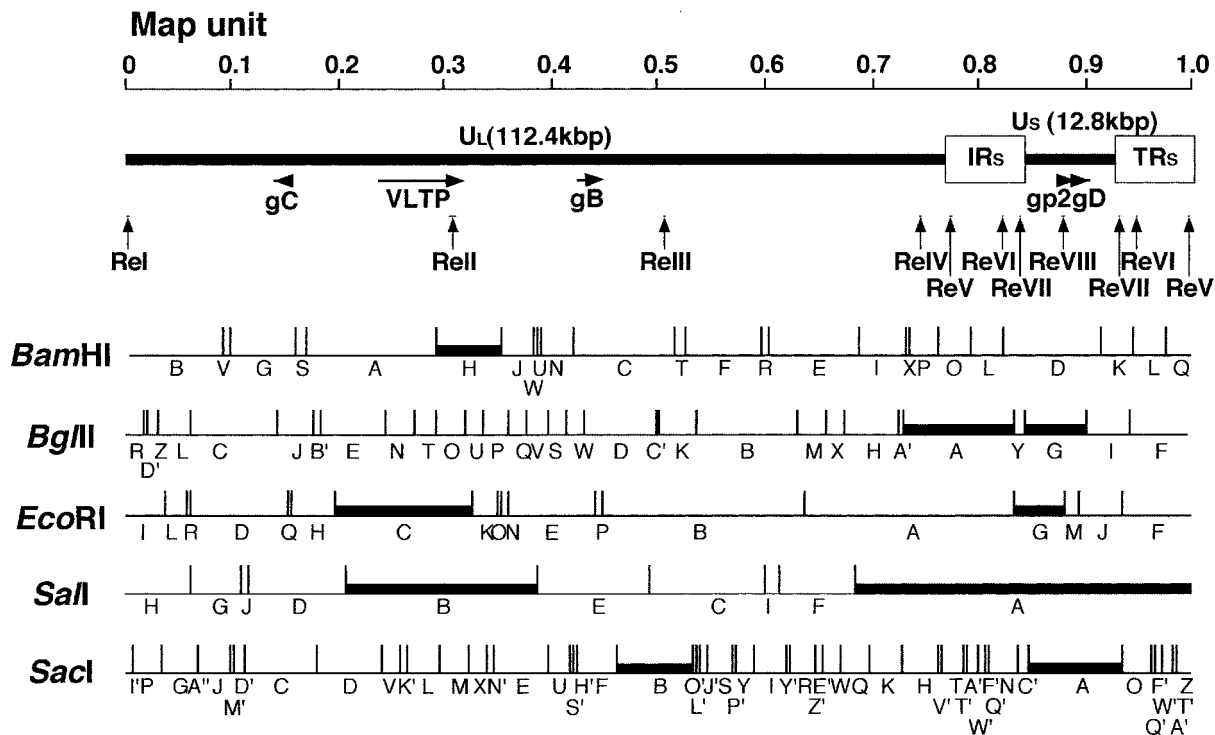

Fig. 3.   Restriction endonuclease map based on the genome of EHV-4 NS80567 strain (Accession No. AF030027). Fragments with mobility shifts among field isolates are shown by black boxes. Eight regions containing reiterations are shown by lines. Open reading frames analyzed in this study are shown by arrows. U_L; unique long region, IR_S; internal repeat region, U_S; unique short region, TR_S; terminal repeat region.
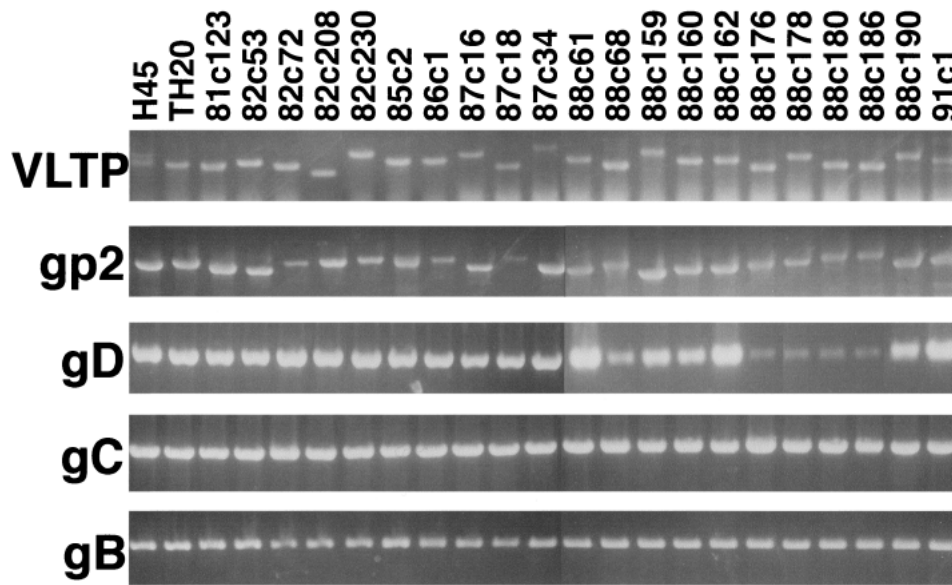
Fig. 4 Comparison of PCR products amplified from EHV-4 field isolates. PCR was carried out as described in Table 2. The amplified fragments were electrophoresed in 1–2% agarose gel and the sizes were compared. The sizes of the amplified fragments were summarized in Table 3.

nucleases *Bam*HI, *Bgl*II, *Eco*RI, *Sac*I, and *Sal*I and eight sequence repeat regions (ReI to ReVIII) are shown in Fig. 3. The H (*Bam*HI), C (*Eco*RI) and B (*Sal*I) fragments that showed mobility shifts contain Re II, the B (*Sac*I) fragment contains ReIII, the A (*Bgl*II) fragment contains Re IV, V and VI, the G (*Eco*RI) fragment contains ReVII and VIII, and the A (*Sac*I) and G (*Bgl*II) fragments contain Re VIII. Therefore, we speculated that changes in sequence repeat regions were responsible for the observed mobility shifts.

Detection of genomic diversity among EHV-4 field isolates by PCR: To detect genomic diversity among EHV-4 field isolates, five sets of PCR primers were designed (Table 2). We focused on two sequence repeats, ReII and ReVIII, because these repeats are contained in the protein-encoding regions, ORFs 24 and 71, of very large tegument protein (VLTP) and gp2, respectively. Three additional genes, encoding the major glycoproteins gB, gC and gD, were analyzed by PCR, because these proteins are expected to be conserved because of their important roles in viral infection. The results indicated variation between the field isolates for ORF24 with ReII and ORF71 with ReVIII, while no diversification was detected for gB, gC and gD (Fig.4 and Table 3). In addition, no difference among epidemiologically related field isolates, 88c160 and 88c162, 88c180 and 88c186, was detected by PCR analysis.

DISCUSSION

In this study, we compared the genomic DNA of 23 EHV-4 Japanese field isolates by restriction endonuclease digestion. The field isolates were differentiated by mobility shifts as well as gain and/or loss of fragments. For example,

*Eco*RI digestion of H45 produced a 22-kbp fragment instead of the 18.5-kbp C (*Eco*RI) and 3.3-kbp K (*Eco*RI) fragments that were observed in TH20. Therefore, the *Eco*RI site at position 47731 between the C and K fragments is likely mutated in H45. This possible mutation is located within ORF26 encoding a membrane-associated phosphoprotein. The *Sal*I digestion pattern of H45 and 88cl76 showed the gain of a 10-kbp fragment instead of the G and J fragments observed in TH20. We speculate that loss of the *Sal*I site at position 16264 between the G and J fragments is responsible for the presence of this 10-kbp fragment. This mutated *Sal*I site is within ORF13 encoding a tegument protein. The origin of the new 6-kbp fragment observed in H45, 81cl23, 82c72, 82c230, and 88cl78 and an approximately 9-kbp fragment in 88c190 following *Sac*I digestion is unclear.

The restriction digest pattern of the Japanese prototype EHV-4 strain H45 was distinctly different from the patterns of the field isolates studied. Both the TH20 and the H45 strain were isolated around 1960; however, the characteristic features of restriction endonuclease digestion pattern in TH20 has maintained to EHV-4 field isolates identified in the 1980's and the UK isolate NS80567. The genomic changes observed in the H45 strain may be explained by the fact that *in vitro* passaging induces changes in the viral genome; however, the TH20 strain has also been propagated *in vitro*. Therefore, we speculate that the changes in the H45 genome have occurred through natural occurring mutations. Although no difference in *in vitro* viral growth and pathogenicity has been demonstrated, the genomic diversity between H45 and the EHV-4 field isolates highlights the special character of H45. Therefore, we suggest that TH20 or one of the other field isolates should be used as Japanese

Table 3. Summary of genomic diversity shown by PCR and restriction endonuclease digestion

Strains and field isolates	PCR		Digestion with restriction endonuclease			
	gp2(kbp)	VLTP(kbp)	*Eco*RI	*Sac*I	*Sal*I	Genotype
H45	2.2	1.15&1.05	22(+)[a], 18.5(–)[b], 3.3(–)	6(+)	10(+), 9.7(–)	E
TH20	2.25	1	–[c]	–	–	A
81c123	2.05	0.95	–	6(+)	–	B
82c53	2.05	1.05	–	–	–	A
82c72	2.3	0.95	–	6(+)	–	B
82c208	2.3	0.85	–	–	–	A
82c230	2.4	1.15	–	6(+)	–	B
85c2	2.3	1	–	–	–	A
86c1	2.4	1	–	–	–	A
87c16	2.15	1.15	–	–	–	A
87c18	2.6	0.9	–	–	–	A
87c34	2.05	1.25	–	–	–	A
88c61	2.15	1.05	–	–	–	A
88c68	2.25	0.9	–	–	–	A
88c159	1.95	1.15	–	–	–	A
88c160[d]	2.05	1	–	–	–	A
88c162[d]	2.05	1	–	–	–	A
88c176	2.25	0.9	–	–	10(+), 9.7(–)	C
88c178	2.25	1.1	–	6(+)	–	B
88c180[e]	2.4	0.9	–	–	–	A
88c186[e]	2.4	0.9	–	–	–	A
88c190	2.15	1.1	–	9(+)	–	D
91c1	2.2	1	–	–	–	A

a) (+) shows the gain of fragments in comparison with TH20.
b) (–) shows the loss of fragments in comparison with TH20.
c) – shows the same pattern as TH20.
d) Epidemiologically related isolates.
e) Epidemiologically related isolates

prototype strain instead of H45.

PCR analysis of genes containing sequence repeats indicated that the number of repeats varied among EHV-4 field isolates. Huang *et al.* [7] also reported that sequence repeats were variable among EHV-4 strains. In EHV-1, changes in the number of repeats within ORFs 1, 24 and 71 were reported after serial passaging in bovine kidney cells [11]. Epizootiologically related isolates, however, did not show any changes in the sequence repeats within ORFs 24 and 71 (Fig.4 and Table 3). Although the number of repeat sequences of herpesviruses is reported to be unsuitable for epidemiological study because of the variability, PCR analysis of sequence repeats in these regions may be a useful tool for the identification of epidemiological relationship among EHV-4 isolates.

In addition to genetic diversity of the EHV-4 ORF71, Huang *et al.* [7] also reported diversity of gp2 encoded by ORF71 at the protein level, indicating that Japanese isolates must also show similar diversity of gp2. Further experiments will be required to clarify the diversity of immunogenic proteins among Japanese isolates. Recently, we also reported the genomic diversity in the *C*-terminal of the EHV-4 ORF70 encoding the gG and the repeat sequences have a unique character that each stretch of repeat sequences encodes different B-cell epitopes with change of one amino acid and a deletion of another amino acid [13]. The significance of the repeat sequences in the EHV-4 gG has been still unknown.

Allen *et al.* [2] reported that 25 American EHV-4 field isolates could be divided into 13 different genotypes based on restriction endonuclease analysis using the same five enzymes, *Bam*HI, *Bgl*II, *Eco*RI, *Sac*I and *Sal*I. Our results indicated that Japanese field isolates could only be divided into five genotypes, genotype A (16 isolates), B (four isolates), C (one isolate), D (one isolate) and E (one isolate) (Table 3). It is not clear whether Japanese isolates are genetically more uniform than American isolates and further investigation using EHV-4 field isolates will be required to elucidate the global diversity among EHV-4 strains.

In this study, the genomic diversity of Japanese EHV-4 strains and field isolates was analyzed by restriction endonuclease digestion. In addition, the epidemiological relationships among field isolates were confirmed by PCR analysis of sequence repeats in ORFs 24 and 71. Based on our findings, variations in restriction endonuclease digestion patterns and the length of sequence repeats appear to be useful markers for the epidemiological study of equine rhinopneumonitis by EHV-4 infection.

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