Spatial and Temporal Genetic Homogeneity of Orf Viruses Infecting Japanese Serows (*Capricornis crispus*)

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ABSTRACT. This study genetically characterized orf viruses (ORFVs) isolated from recent outbreaks in Japanese serows (*Capricornis crispus*) in 2007 and 2008, and from earlier outbreaks from 1985 to 2001. Nucleotide sequences of genes for the viral envelope, vascular endothelial growth factor (VEGF), and virus interferon resistance (VIR) were determined in two ORFVs isolated from recent outbreaks and in eight from earlier outbreaks. No deletions or insertions were observed in these genes. Surprisingly, the amino acid sequences of the envelope and VIR genes were identical among the 10 ORFVs, and only one amino acid substitution in the VEGF gene was found in one of the two recent ORFVs (2007). Genotyping by differential PCR for the A32L gene, which encodes an ATPase, classified all of the 10 ORFVs into the same genotype. In an ORFV isolated from a sheep in 1970, the three sequenced genes were almost the same as in the ORFVs isolated from Japanese serows, and the ORFV in 1970 was classified into the same genotype as the ORFVs from Japanese serows. These results suggest that recent outbreaks in Japanese serows are caused by ORFVs genetically closely related to the viruses in earlier outbreaks and that these genetically stable ORFVs have circulated among Japanese serows over a long period of time.

KEY WORDS: contagious pustular dermatitis, Japanese serow, orf virus, outbreak, sequence.

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Parapoxviruses (PPVs) cause papular stomatitis and pseudocowpox in cattle and contagious pustular dermatitis in sheep and goats [8, 33]. PPV infections in Japan were reported in cattle in the 1960s [32], in sheep in the 1940s [2] and in wild Japanese serows (*Capricornis crispus*) in the 1970s [26]. Serological surveys have demonstrated that 72.0% of cattle (1,310/1,819) from various regions of Japan were seropositive for PPV, ranging from 40.0 to 98.0% in 1998 [36] and that 88.5% of sheep in Hokkaido were seropositive [27]. Seroprevalence among Japanese serows was previously reported to be 24.0% (40/167 from 1984 to 1999) [20, 21]. These findings indicate that healthy cattle and sheep can be infected subclinically with PPV and that the infection is widespread among both domestic and wild ruminants in Japan.

Recently, PPV epidemics have been frequently observed in wild Japanese serows in central Japan. Epidemics have occurred in these areas since December 1984, and 155 of 402 serows (39.6%) captured from December 1984 to March 1985 were clinically affected [40]. Tikkanen *et al.* reported that a recent outbreak of PPV infection in Finnish reindeer (*Rangifer tarandus tarandus*; 1999 to 2000) was caused by pseudocowpox virus, a species of the genus *Parapoxvirus*, whereas an earlier outbreak (1992 to 1994) was caused by orf virus (ORFV), a prototype species of PPV [42]. Sequence analyses of the viral envelope gene indicate that contagious pustular dermatitis in Japanese serows is mainly caused by ORFV [18, 19]. However, only a partial sequence of the envelope gene was analyzed in these stud-

ies, and in other countries, sequence variations occur in the envelope gene of ORFVs isolated from the same outbreaks [1, 5, 6, 12, 14, 24]. In general, the amino acid sequences of the genes encoding viral structural proteins, such as the envelope, are relatively well conserved, but the detailed genetic relationship between the PPVs of the recent and earlier outbreaks in Japanese serows is unclear. To assess genetic heterogeneity among PPVs in Japanese serows, it is necessary to analyze other genes, especially genes encoding non-structural proteins.

In this study, we analyzed nucleotide sequences of three viral genes that encode both structural and non-structural proteins, namely, the viral envelope, vascular endothelial growth factor (VEGF) and virus interferon resistance (VIR) genes. The VEGF and VIR genes are thought to be among the several virulence or immune modulator genes [13]. We also genotyped PPVs isolated from recent and earlier outbreaks in Japanese serows using differential PCR for the A32L gene [4], which encodes an ATPase and is involved in DNA packaging [9, 30].

MATERIALS AND METHODS

Viruses and cells: Two PPVs from recent outbreaks in Japanese serows (2007 and 2008) and eight PPVs from earlier outbreaks (1985 to 2001) were used in this study. We used both isolated PPVs and lesions of affected animals for genetic analyses because virus isolation is sometimes unsuccessful even though virus-like particles are observed in the lesions and it is also possible that a genetically minor virus can be selected and propagated in cell culture and isolated. Three ORFVs from sheep in Japan were also used for comparison. The virus source locations are shown in Fig. 1,

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Fig. 1. Source locations of PPVs from Japanese serows and sheep. The year of isolation or collection is also listed. (A) Hokkaido, (B) Iwate, (C) Fukushima, (D) Nagano, (E) Toyama, (F) Ishikawa, (G) Gifu and (H) Aichi Prefectures.

and other information, including sequence accession numbers, is shown in Table 1. The GE (Gifu Ena) strain was newly isolated in this study from scab lesions around the mouth of an affected Japanese serow in 2007 and was passaged twice in primary fetal lamb lung cells. The IJS081 strain was isolated from lesions of the lips of an affected Japanese serow in 2008 [22]. These two strains were used as PPVs from recent outbreaks. All PPVs were propagated in primary fetal bovine muscle cells. Cells were maintained in Eagle's minimum essential medium (MEM, Nissui,

Tokyo, Japan) supplemented with 0.3% tryptose phosphate broth (Difco, Detroit, MI, U.S.A.), 10% fetal bovine serum and 60 mg/l kanamycin.

DNA extraction: DNA was extracted from virus-infected cells or directly from lesions using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Polymerase chain reaction (PCR) and sequencing: The primers used for PCR and sequencing are shown in Table 2. PCR conditions for envelope, VEGF and VIR were as follows. The PCR reaction mixture (50 μ l) contained 0.2 μ M each of primer, 800 μ M dNTP mix, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, U.S.A.) and 1 μ l extracted DNA. PCR consisted of 9 min at 95°C followed by 35 cycles at 94°C for 30 sec, 55°C for 30 sec (40°C for VEGF) and 72°C for 30 sec, with a final extension at 72°C for 7 min. For sequencing, the PCR products were purified using a QIAquick PCR Purification Kit (Qiagen), and nucleotide sequences were determined by direct sequencing using an ABI 310 DNA sequencer with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). For verification, sequencing was performed in both directions.

The genotyping of ORFVs by differential PCR for the A32L gene, based on variations and deletions in the sequence, was recently described by Chan *et al.* [4]. The PCR conditions for A32L were as follows. The PCR reaction mixture (50 μ l) contained 1 μ M FP primer, 0.4 μ M RP1 primer, 0.4 μ M RP2 primer, 800 μ M dNTP mix, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 1 U AmpliTaq Gold DNA polymerase and 3 μ l extracted DNA. PCR consisted of 9 min at 95°C followed by 35 cycles at 94°C for 30 sec, 63°C for 30 sec and 72°C for 30 sec, with a final extension at 72°C for 7 min. DNAs from Taiwanese ORFV Nantou, Taiping and Hoping strains [4] were used as the standard strains in the genotyping, and DNA from the

Table 1. Viruses and accession numbers for the sequences

Host	Virus	Reference	Accession No.*				
			Envelope	VEGF	VIR		
Japanese serow	Isolate	IJS081	22	AB492240	AB492084	AB492085	
		GE	This study	AB493826	AB436811	AB499037	
		Aichi	16,18	AB521165	AB436810	AB522795	
		GHF	16,18	AB521166	AB436809	AB522796	
		R-1	16,18	AB521167	AB436808	AB522797	
		S-1	16,19,39	AB044796	AB436807	AB492086	
	Tissue	Kohriyama	18	AB521169	AB522648	AB522799	
		Suzuran	18	AB521171	AB522649	AB522800	
		Iwamura	18	AB521172	AB522650	AB522801	
		Matsumoto	18	AB521174	AB522651	AB522802	
	Sheep	Isolate	HIS	16,23	AB189670	AB436806	AB522798
			Iwate	16,19,25	AB044795	AB436805	AB499038
Tissue		Ena	18	AB521175	AB522652	AB522803	

* VEGF, vascular endothelial growth factor. VIR, viral interferon resistance.

Table 2. Primers used in this study

Primer	Target*	Sequence (5' – 3')**	Reference
PPP-1	Envelope	GTC GTC CAC GAT GAG CAG CT	17
PPP-4		TAC GTG GGA AGC GCC TCG CT	
D27F	VEGF	AAT GTA AAT WMT AAC GCC	16
11R		AAC CCA GAA ACG TCC CGC TAC	
D2R		CGT TTG GAT MTG CGG TCC	
VIR1	VIR	TTA GAA GCT GAT GCC GCA G	12
VIR2		ACA ATG GCC TGC GAG TG	
FP	A32L	GTG TTG ATC ATC GAA GAC TCG GTG	4
RP1		GTC GCC CTT GTC GCC CTT AGT CTC	
RP2		CCG CCG TCA GAG TCG ACG TCG CCC T	

* VEGF, vascular endothelial growth factor. VIR, virus interferon resistance.

** W, A/T. M, A/C.

ORFV NZ2 strain [34] was used as a reference strain. DNA from the Taiwanese ORFV and NZ2 strains were kindly provided by Drs. M. L. Wong (National Chung-Hsing University, Taiwan) and A. A. Mercer (University of Otago, New Zealand), respectively.

RESULTS

Sequence analyses: A partial nucleotide sequence of the viral envelope gene (554 bp) was the first sequence determined from the PCR product. From the sequence analysis, the newly isolated GE (2007) and IJS081 (2008) strains were classified as ORFVs. This indicates that the recent outbreaks in Japanese serows were caused by ORFV and not by other PPV species, as in the case of the recent reindeer outbreak [42]. The sequences from both the isolated virus and lesions were identical, indicating that this was not a case of isolation of a genetically minor virus from lesions. The GE and IJS081 sequences had no deletions or insertions compared with the published sequences of the S-1 strain, which was isolated in 1985, and compared with sequences from the ORFVs of earlier outbreaks in Japanese serows [18,19]. The only difference was a single nucleotide substitution in the GE sequence compared with the S-1 sequence, which had no changes in the coded amino acid (Fig. 2A).

The complete nucleotide sequences of the VEGF and VIR genes were determined to be the same length among all 10 ORFVs that infected Japanese serows, i.e., 137 and 183 amino acids, respectively (Fig. 2B and 2C). The VEGF sequences in the S-1 and IJS081 strains differed by one amino acid substitution, whereas the S-1 and GE sequences were identical; the VEGF sequences of S-1, Aichi, GHF, R-1, Suzuran, Iwamura and Ena were also identical. There was one amino acid substitution in the VEGF sequences of the Kohriyama, Matsumoto and Iwate strains, which was at a different position compared with the substitution in the IJS081 sequence. However, in comparison with the S-1 sequence, there were no more than two amino acid substitutions in the VEGF sequences among the ORFVs from Japanese serows and sheep, with the exception of the HIS strain from sheep. The amino acid sequence of HIS VEGF was quite different from the other VEGF sequences; however,

eight cysteine residues in the gene were conserved among all of the Japanese ORFVs, including the HIS strain as described previously [16]. These conserved residues are also conserved in all members of the VEGF family, including viral VEGF, human VEGF-A to -D, placenta growth factor 1 and bovine, guinea pig and rat VEGFs [28, 31].

The amino acid sequence of the VIR gene was identical among all 10 ORFVs from Japanese serows and two of the ORFVs from sheep (Fig. 2C), with no nucleotide substitutions. The only differences were in the sequence from the HIS strain from sheep, which contained two amino acid substitutions. The determined nucleotide sequences were submitted to DDBJ/EMBL/GenBank under the accession numbers shown in Table 1.

Genotyping by differential PCR for the A32L gene classified all 13 Japanese ORFVs into the same genotype (Fig. 3). The genotype of the Japanese ORFVs is the same as that of the Taiping and NZ2 strains (Fig. 3).

DISCUSSION

The sequence of the PPV envelope gene, a homologue of vaccinia virus major envelope antigen p37K [38], has been widely used as a molecular target for virus detection and diagnosis of infection [11, 17] and for identification of species within the genus *Parapoxvirus* [3, 19, 41]. Since a large number of sequence data sets have been deposited in DDBJ/EMBL/GenBank, they can be used for epidemiological and phylogenetic analyses of PPVs. Thus, to characterize the PPVs in recent outbreaks (2007 and 2008) in Japanese serows, we first determined the sequence of the envelope gene.

Previous sequence analyses of the envelope gene revealed that the sequence is highly conserved among Japanese ORFVs [18, 19]. The amino acid sequences of the newly isolated GE and IJS081 strains were also almost identical to the previously determined sequences, containing only one nucleotide silent mutation in the GE strain. These results suggest that the envelope sequence is highly conserved in ORFVs infecting Japanese serows. However, our results are not consistent with observations in other countries, where sequence variations in the envelope gene of

(A) Envelope

S-1	1: TGGSVSTIKNLGLYSTKNKHLAWDLNMRYNTFYSMIVEPKVPFTRLCCAVVTPTATNFHLDHSGGGVFFSDSPERFLGFYRTLDEDLVLRHENAKNSIDL	100
IJS081	1:.....	100
GE	1:.....	100
Aichi	1:.....	100
GHF	1:.....	100
R-1	1:.....	100
Kohriyama	1:.....	100
Suzuran	1:.....	100
Iwamura	1:.....	100
Matsumoto	1:.....	100
Iwate	1:.....	100
Ena	1:.....	100
HIS	1:.....	100
S-1	101: SLLSMVPIKHASAVEYWPRIIDALLRAAIDRGVRVRIITEWKNADPLSVSAARSLLDFGVGSVDM SVRK FV VPGRDDAANNT	184
IJS081	101:.....	184
GE	101:.....	184
Aichi	101:.....	184
GHF	101:.....	184
R-1	101:.....	184
Kohriyama	101:.....	184
Suzuran	101:.....	184
Iwamura	101:.....	184
Matsumoto	101:.....	184
Iwate	101:.....	184
Ena	101:.....	184
HIS	101:.....	184

(B) VEGF

S-1	1: MRLLCVILVVVCLLHQHLNADSSSTKKWEVLEGSCKKPRPTVLSVNGEHPELTSQRFPNPPCVTLMRCCGCCNDESLECVPTEEANVTMEFMGVGVSSSTG	100
IJS081	1:.....S.....	100
GE	1:.....	100
Aichi	1:.....	100
GHF	1:.....	100
R-1	1:.....	100
Kohriyama	1:.....	100
Suzuran	1:.....	100
Iwamura	1:.....	100
Matsumoto	1:.....	100
Iwate	1:.....	100
Ena	1:.....	100
HIS	1:.....G...A...Y...-...T.S...F.S...VP.GEA...Q...V...QL...AS...G-..	97
S-1	101: SSVSTQHLEFVEHTKDCQPRGGQQTTPPRRRRAY	137
IJS081	101:.....	137
GE	101:.....	137
Aichi	101:.....	137
GHF	101:.....	137
R-1	101:.....	137
Kohriyama	101:.....P.....	137
Suzuran	101:.....	137
Iwamura	101:.....	137
Matsumoto	101:.....P.....	137
Iwate	101:.....P.....	137
Ena	101:.....	137
HIS	98:NGM--...I...K...K...LTTTPP.TT.PP..RR	132

ORFVs from the same outbreak have been reported [1, 5, 6, 12, 14, 24]. Guo *et al.* reported an outbreak of ORFV infection in a zoo, in which a single ORFV likely infected musk ox (*Ovibos moschatus*), domestic Shetland sheep (*Ovis aries*) and Sichuan takin (*Budorcas taxicolor tibetana*), but the amino acid sequence of the viral envelope gene contained one or two substitutions in each animal [12]. These contradictory findings raise the possibility that, although the envelope sequence is suitable for species-identification and phylogenetic analysis of PPVs, it is not suitable for analysis of genetic heterogeneity in Japanese ORFVs. Therefore, we analyzed further gene sequences and genotyped ORFVs using three non-structural protein genes, VEGF, VIR and A32L.

The VEGF and VIR proteins are viral non-structural pro-

teins known to be factors involved with virulence and interference of host immune responses [13]. Skin lesions and histopathological changes were relatively reduced in sheep experimentally infected with ORFV lacking functional VEGF [35, 43]. The VIR gene encodes a dsRNA-binding protein that is involved in inhibition of the antiviral activity of host interferons [29]. Surprisingly, the amino acid sequences of the VEGF and VIR genes of the GE strain were identical to the sequences of the S-1 strain, which was isolated in 1985, and also to the VIR sequence of the Iwate strain, which was isolated in 1970. One amino acid was substituted in the IJS081 VEGF sequence, but the IJS081 VIR sequence is identical to the S-1 and Iwate sequences. Chan *et al.* similarly observed only a few variations in the envelope and VIR gene sequences in Taiwanese ORFVs

(C) VIR

S-1	1:MAC	100
IJS081	1:.....	100
GE	1:.....	100
Aichi	1:.....	100
GHF	1:.....	100
R-1	1:.....	100
Kohriyama	1:.....	100
Suzuran	1:.....	100
Iwamura	1:.....	100
Matsumoto	1:.....	100
Iwate	1:.....	100
Ena	1:.....	100
HIS	1:.....	100
S-1	101:AVIRLKS	183
IJS081	101:.....	183
GE	101:.....	183
Aichi	101:.....	183
GHF	101:.....	183
R-1	101:.....	183
Kohriyama	101:.....	183
Suzuran	101:.....	183
Iwamura	101:.....	183
Matsumoto	101:.....	183
Iwate	101:.....	183
Ena	101:.....	183
HIS	101:.....S...A.....	183

Fig. 2. Alignment of the deduced amino acid sequences. (A) Partial amino acid sequences of the viral envelope gene. (B) Complete amino acid sequences of the VEGF gene. (C) Complete amino acid sequences of the VIR gene.

Fig. 3. Genotyping of Japanese ORFVs by differential PCR for the A32L gene. Three standard strains of genotypes showed three different patterns (Lane 1, Nantou, Lane 2, Taiping, and Lane 3, Hoping). All of the Japanese ORFVs (Lanes 5 to 17) were classified into the same genotype with the Taiping (Lane 2) and NZ2 (Lane 4) strains. Lane M, Marker. Lane 4, NZ2 as a reference strain. Lane 5, IJS081. Lane 6, GE. Lane 7, Aichi. Lane 8, GHF. Lane 9, R-1. Lane 10, S-1. Lane 11, Kohriyama. Lane 12, Suzuran. Lane 13, Iwamura. Lane 14, Matsumoto. Lane 15, HIS. Lane 16, Iwate. Lane 17, Ena. Lane DW, distilled water as a negative control.

from goats [4]. All 13 Japanese ORFVs were classified into the same genotype by the differential PCR for the A32L gene. On the other hand, the Taiwanese ORFVs were split into three genotypes by the differential PCR for the A32L gene, indicating sequence variations and deletions in the gene [4], and genome recombination, such as duplication, transposition and deletion, in cultured cells has also been reported [7, 10]. Thus, these results suggest that ORFVs that have a genetic character like the S-1/Iwate strain other than the HIS strain, at least in the envelope, VEGF and VIR genes, are highly adapted and maintained in Japanese serows. These factors may be involved in the adaptation to Japanese serows, namely receptor-usage, interaction with cellular proteins for viral replication and interference with the host immune responses.

In conclusion, comparative genetic analyses revealed that recent outbreaks in Japanese serows are caused by ORFVs that are genetically closely related to the ORFVs found in

earlier outbreaks and that these ORFVs have been circulating among Japanese serows over a long period. It is possible that, like cattle with bovine papular stomatitis virus [15, 37], certain Japanese serows are subclinically infected with ORFVs and that relapse of the disease occurs.

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