Pseudorabies virus infection in hunting dogs in Oita, Japan: Report from a prefecture free from Aujeszky's disease in domestic pigs

Chiho KANEKO[1], Yasuyuki KANEKO[2], Putu Eka SUDARYATMA[3], Hirohisa MEKATA[4], Yumi KIRINO[1,2], Ryoji YAMAGUCHI[2] and Tamaki OKABAYASHI[1,2]*

[1]Centre for Animal Disease Control, University of Miyazaki, 1-1 Gakuen Kibanadai-nishi, Miyazaki 889-2192, Japan
[2]Department of Veterinary Science, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen Kibanadai-nishi, Miyazaki 889-2192, Japan
[3]Graduate School of Medicine and Veterinary Medicine, University of Miyazaki, 5200 Kiyotakecho Kihara, Miyazaki 889-1692, Japan
[4]Organization for Promotion of Tenure Track, University of Miyazaki, 1-1 Gakuen Kibanadai-nishi, Miyazaki 889-2192, Japan

ABSTRACT. We isolated two pseudorabies virus (PRV) isolates (designated OT-1 and OT-2) from two hunting dogs exhibiting neurological manifestations after eating the flesh of wild boar hunted in Oita prefecture, Kyushu Island, Japan. The isolates corresponded to a previously reported PRV (MY-1 strain) isolated from a hunting dog in neighboring Miyazaki prefecture, and it clustered into genotype II based on the glycoprotein C sequence. Our results suggest that this common PRV strain may have been maintained in wild boars on Kyushu Island even though domestic pigs in this area have attained an Aujeszky's disease-free status.

KEY WORDS: Aujeszky's disease (AD), hunting dog, pseudorabies virus (PRV), wild boar

Pseudorabies virus (PRV), also known as suid alphaherpesvirus 1, is the causative agent of Aujeszky's disease (AD), which affects mainly pigs and wild boars [14, 21]. It is a DNA virus belonging to the genus *Varicellovirus*, subfamily *Alphaherpesvirinae*, and family *Herpesviridae* [24]. AD causes huge economic losses, and clinical signs of infected pigs include respiratory distress, neurological manifestations, and abortion depending on the age at which animals are exposed [9, 14]. Even if animals survive, latent virus persists in their body as a so-called reservoir, and the virus continues to circulate among pig and wild boar populations [9, 21, 22].

The first reported case of AD in Japan was in pigs reared in Yamagata prefecture in 1981 [11], after which the disease spread to most of the other prefectures. The Japanese Ministry of Agriculture, Forestry and Fisheries established a control program against AD in 1991. As a result of the highly successful control program involving vaccination strategies that followed DIVA ("differentiating infected from vaccinated animals") [3], PRV was gradually eradicated from the prefectures, and an AD-free status has been attained in all but one prefecture as of March 31, 2020.

In animals other than pigs, PRV infection causes fatal encephalomyelitis accompanied by neurological signs, such as pruritus and convulsions, resulting in death within a few days of the onset of clinical signs [9, 23]. The first case of AD in animals other than pigs in Japan was observed in cattle in 1985 [19]. Subsequently, AD has been reported in dogs [7, 12, 18] and a cat [8]. The aforementioned cases of PRV infection in dogs were suspected to be caused by the consumption of raw meat or offal from PRV-infected pigs or through direct contact with infected pigs. Recently, Minamiguchi *et al.* isolated PRV from a hunting dog that had bitten a wild boar in Miyazaki prefecture, Japan [20]. In addition, Mahmoud *et al.* reported that the wild boar population in western Japan carried PRV-neutralizing antibodies even though pigs in this area are considered PRV-free [16]. These reports indicate the presence of PRV in wild boars in Japan. This study describes cases of suspected AD in hunting dogs that exhibited clinical signs after wild boar hunting in Oita prefecture, a prefecture that is designated as AD-free.

Two 5-year-old mixed breed dogs (one female [designated dog 1] and one male [designated dog 2]) in Oita, Japan became critically ill in March 2018. The dogs had consumed wild boar meat after hunting on March 3, 2018. They exhibited depression and anorexia on the first day of illness (March 5, 2018). On the third day of illness, they started to display atypical behavior that

was characterized by motor incoordination, rubbing of their faces against surfaces (e.g., surfaces of objects/structures or walls) because of severe pruritus, and lunging. Clinical signs including an inability to walk persisted, and death occurred 4 days after the first observation of clinical signs (March 8, 2018). The hunting dogs had been vaccinated with an eight-valent vaccine against canine distemper, canine parvovirus infection, canine adenovirus infections (type-1 and type-2), canine parainfluenza, canine coronavirus infection, and leptospirosis (two strains).

Postmortem examination was conducted, and tissue samples (trigeminal nerve, bone marrow, lungs, spleen, and serum from dog 1 and trigeminal nerve, medulla oblongata, bone marrow, lungs, spleen, and kidneys from dog 2) were collected. Each of the samples was ground in Eagle's minimum essential medium (MEM; Sigma-Aldrich, St. Louis, MO, USA) in sterilized 1.5-ml tubes with disposable pestle sticks to create 7% suspensions. The samples were centrifuged at 9,300 ×g for 5 min, and supernatants were used for virus isolation.

For virus isolation, Vero cells maintained with MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biowest, Nuaillé, France), 100 units/ml penicillin, and 100 µg/ml streptomycin (FUJIFILM Wako Pure Chemical, Osaka, Japan) were used. The cell density was adjusted to 1×10^5 cells/ml, and cells were seeded in six-well plates and incubated for 24 hr. In total, 600 µl of the supernatant of the 7% homogenate of each tissue sample was inoculated onto Vero cells. After adsorption for 90 min, the inoculum was removed, cells were washed with phosphate buffered saline, and MEM containing 2% heat-inactivated FBS was added. For each sample, a blind passage incubation was completed every seventh day at 37°C and 5% CO_2 and continued until the third passage. Cytopathic effects were observed in Vero cells inoculated with different tissue samples from each dog. These were characterized by the diffuse degeneration of the cell monolayer, with rounded cells floating within the culture medium after three passages. Cells inoculated with the samples derived from trigeminal nerve, bone marrow, lung, and spleen tissues from dog 1 exerted obvious cytopathic effects, as did cells inoculated with samples derived from medulla oblongata, bone marrow, and lung tissues from dog 2. Each supernatant from the third blind passage was stored at −80°C until use for virus identification.

Viral DNA/RNA was extracted from the aforementioned supernatants using the NucleoSpin Virus (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. PCR was performed to detect PRV using the extracted DNA. RT-PCR was performed using the extracted RNA to detect other pathogens, namely rabies lyssavirus (RABV) for rabies, dabie bandavirus (SFTSV) for severe fever with thrombocytopenia syndrome, and canine morbillivirus (CDV) for canine distemper. The primer sets are presented in Table 1. The positive controls for PCR/RT-PCR were as follows: DNA of the PRV Begonia strain extracted from the Porcilis Begonia DF·10 vaccine (MSD Animal Health K. K., Tokyo, Japan), RNA of the RABV challenge virus standard (CVS) strain cultured in NA cells, double-stranded DNA artificially synthesized according to the nucleotide sequence of the SFTSV YG1 strain (GenBank accession no: AB817995), and RNA of CDV (strain Onderstepoort) extracted from the Nobivac Puppy DP vaccine (MSD Animal Health K. K.). We conducted PCR targeting the glycoprotein C (gC, UL44) gene of PRV in a 25.0-µl reaction mixture containing 12.5 µl of 2× Gflex PCR Buffer (Mg^{2+}, dNTP plus, Takara Bio, Kusatsu, Japan), 0.5 µl of Tks Gflex DNA Polymerase (1.25 units/µl, Takara Bio), 30 µM of the primer set (final concentration: 0.3 µM each), and 1.0 µl of template DNA. The reaction conditions were as follows: 94°C for 1 min, denaturation at 98°C for 10 sec, annealing at 60°C for 15 sec, and extension at 68°C for 30 sec. The samples were subjected to 30 cycles of amplification and maintained at 68°C for 7 min. The RT-PCR reaction for RABV was conducted following the previously reported method [10]. The RT-PCR reaction for SFTSV was conducted following the method described by Takahashi et al. [27]. The RT-PCR reaction for CDV was performed as described by Demeter et al. [2]. The PCR/RT-PCR products were observed in 1% agarose gel (Nacalai Tesque, Kyoto, Japan) stained with GelRed (Biotium, Fremont, CA, USA). The result illustrated that PCR amplification targeting a partial sequence of the gC gene of PRV identified amplicons with an expected size of 927 bp using culture supernatants derived from the cells cultured with trigeminal nerve, bone marrow, lung, and spleen tissues from dog 1 and medulla oblongata, bone marrow, and lung tissues from dog 2. In the other samples (serum from dog 1; trigeminal nerve, spleen, and kidneys from dog 2), PCR-amplified bands were not observed. RT-PCR assays for RABV, SFTSV, and CDV were negative.

An indirect immunofluorescence assay (IFA) was used to confirm the presence of infectious PRV in supernatants from the third blind passage. Tissue culture-treated glass slides mounted with four polystyrene chambers (Corning, New York, NY, USA) were seeded with 1×10^5 Vero cells/well. Each chamber of the slide was inoculated with four-fold diluted supernatant from the third blind passage (50 µl of supernatant diluted with 150 µl of MEM containing 2% heat-inactivated FBS) and incubated at 37°C in an atmosphere of 5% CO_2. At 15 hr after inoculation, cells were fixed in 4% paraformaldehyde phosphate buffer solution (FUJIFILM Wako Pure Chemical) for 20 min. Cells were permeabilized with 0.2% Triton X-100 (Nacalai Tesque) and blocked with 1% Block Ace (KAC, Kyoto, Japan) for 30 min at room temperature. Fixed cells were stained with rabbit anti-pseudorabies virus polyclonal antibody (ab3534; Abcam, Cambridge, UK) at 1.0 µg/ml (1:1,000) for 30 min followed Alexa Fluor 488-conjugated goat anti-rabbit IgG polyclonal antibody (ab150077; Abcam) at 2.1 µg/ml (1:1,000) for 15 min at room temperature. Cells were covered with 70% glycerin and examined via fluorescence microscopy (BZ-9000; Keyence, Osaka, Japan). The trigeminal nerve, bone marrow, lung, and spleen samples from dog 1 were positive for PRV antigens. The medulla oblongata, bone marrow, and lung samples from dog 2 were also positive for PRV antigens. PRV antigens were not detectable in the other samples. The two PRV isolates in this study were named OT-1 (from dog 1) and OT-2 (from dog 2).

Sequencing was performed via dye terminator sequencing based on the Sanger method using a capillary DNA sequencer. The PCR products of the isolated virus were purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and 3130 Genetic Analyzers (Applied Biosystems) according to the manufacturers' instructions. Phylogenetic analysis of the partial sequence of the PRV gC gene and several sequences of PRV gC available in GenBank was conducted. The sequences were aligned using the ClustalW

Table 1. Primer sets used for PCR/RT-PCR amplification in this study

| Target pathogen	Primer name		Sequence (5′ to 3′)	Target region	Annealing temperature (°C)	Fragment size (bp)	Reference
Pseudorabies virus	PrV gC Forward		CCGACCCCGAGTACTTTGAC	gC gene	60	927	This study
	PrV gC Reverse		TGATCATGCTGGTCACGACG				
Rabies lyssavirus	N7 (mix)		ATGTAACACCYCTACAATGG	N gene	55	606	[10]
	JW6 (mix)	JW6 (DPL)	CAATTCGCACACATTTTGTG				
		JW6 (E):	CAGTTGGCACACATCTTGTG				
		JW6 (M):	CAGTTAGCGCACATCTTATG				
Dabie bandavirus	Forward SFTSV NP-1F		ATCGTCAAGGCATCAGGGAA	NP gene	52	458	[27]
	Reverse SFTSV NP-1R		TTCAGCCACTTCACCCGAA				
Canine morbillivirus	Forward		AACTTAGGGCTCAGGTAGTC	H gene	56	2,023	[2]
	Reverse		AGATGGACCTCAGGGTATAG				
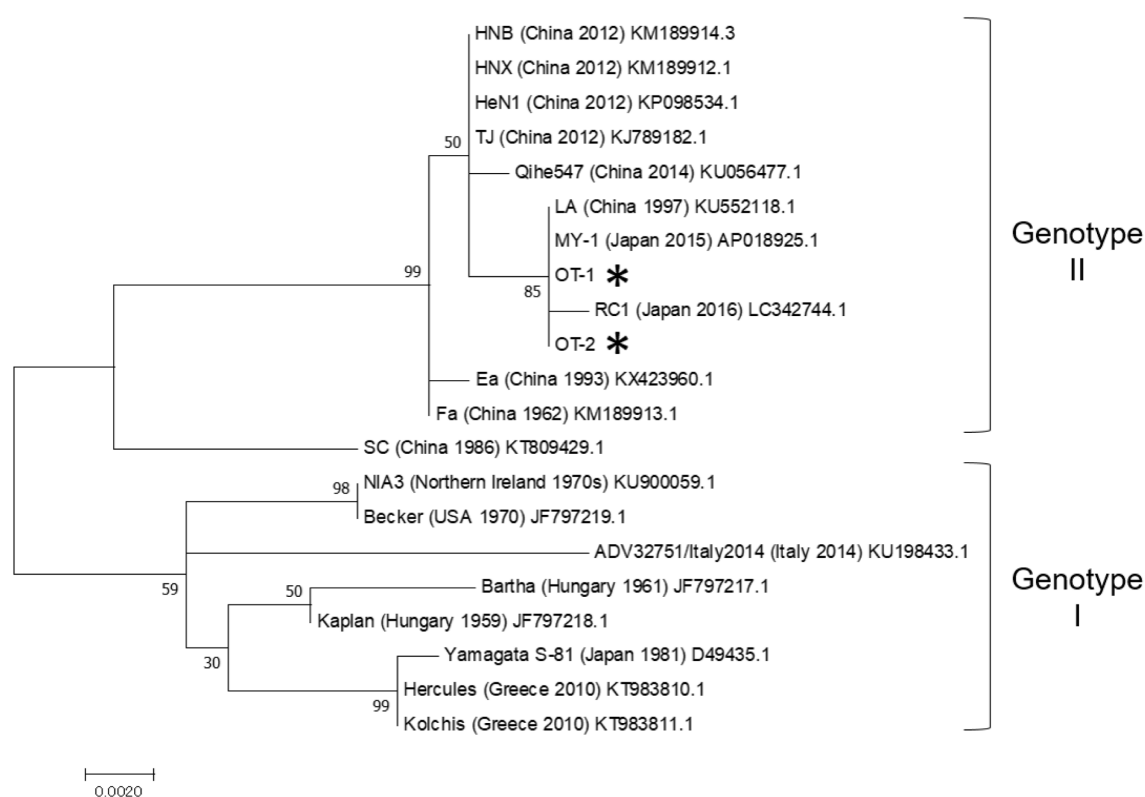
Fig. 1. Phylogenetic analyses based on the partial nucleotide sequences of the pseudorabies virus (PRV) glycoprotein C (gC, UL44) gene. A phylogenetic tree was constructed using the partial 927-bp nucleotide sequences of the gC gene of OT-1, OT-2, and other strains isolated globally. Genotype I consists of PRV strains isolated from European and American countries, as well as a PRV strain isolated in Japan in 1981. Genotype II consists of PRV strains isolated from China and Japan (since 2015). The scale bar indicates the number of substitutions per site. All bootstrap values from 1,000 replications are presented on the corresponding nodes. The two isolates identified in this study are OT-1 (GenBank accession no. LC570808) and OT-2 (LC570809). Asterisks (*) denote the PRV isolates in this study.

protocol [29], and a phylogenetic tree was constructed using the maximum likelihood method based on the Tamura 3-parameter model [28] with 1,000 bootstrap replications in MEGA 7.0 software [13]. The sequences of the gC gene (927 bp) from OT-1 and OT-2 each shared 100% (927/927) nucleotide and 100% (308/308) amino acid homology with MY-1 (AP018925), a PRV isolated in Miyazaki prefecture, Japan in 2015 [20]. Compared with the RC1 (LC342744) strain, which was isolated in Japan in 2016, the gC sequences from OT-1 and OT-2 each shared 99.9% (926/927) nucleotide and 99.7% (307/308) amino acid homology with RC1. The partial nucleotide sequences for the gC genes of PRV isolates (OT-1 and OT-2) in this study were deposited in the DDBJ/EMBL/GenBank databases under the accession numbers LC570808 (for OT-1) and LC570809 (for OT-2). In the phylogenetic analysis, OT-1 and OT-2 clustered into the same group as MY-1 and RC1 based on the 927-bp partial sequence of the gC gene (Fig. 1). This group contains several PRV strains recently isolated in China that have been designated "genotype II" based on

the phylogenetic classification of the gC gene [33, 34]. Strains/isolates belonging to the genotype II are distinct from European-American strains, as well as the Yamagata S-81 strain isolated in Japan, all of which belong to genotype I.

In this study, PRV infection in two hunting dogs in Oita, Japan was confirmed by virus isolation, IFA, PCR, and sequencing. The dogs had eaten fresh meat from wild boars 2 days before the onset of clinical signs, and therefore, it is likely that wild boar flesh was the source of PRV infection. These hunting dogs resided in a prefecture in which AD has been eradicated from domestic pigs. This strongly suggests that PRV can exist in the wild boar population even if the domestic pig population in a prefecture is free from AD. Wild boars would thus represent a reservoir for PRV and risk factors for the transmission of PRV to domestic pigs [6, 21, 30] and other animals including hunting dogs [5, 17, 20–23, 25, 26]. The PRV isolates identified in this study were clustered into "genotype II," based on the nucleotide sequence of the gC gene (gC-genotype II) [20, 33, 34]. Because the Yamagata S-81 strain isolated from domestic pigs, the first reported PRV strain in Japan [4], is known to be clustered into "gC-genotype I" [33], it is suggested that invasion by different genotypes of PRV strains has occurred in Japan. gC-genotype II consists primarily of variant PRV strains that were recently isolated in China [1, 15, 32] and some old Chinese strains [31, 35], as well as the recently reported Japanese PRV strains MY-1 and RC1. Therefore, it was proposed that these strains should be grouped as Asian type PRV [20]. MY-1 was isolated from a hunting dog in Miyazaki prefecture, which is a neighboring prefecture of Oita, on Kyushu Island, Japan in 2015 [20], and RC1 was isolated from a raccoon (*Procyon lotor*) in Japan in 2016. This indicates that it is possible that wild boar populations in Kyushu Island have shared at least one common PRV strain, and such a situation may have also occurred in other areas of Japan, implying the possibility that other Asian type PRV strains might have been maintained in wild boar populations.

CONFLICT OF INTEREST. The authors declare no conflicts of interest associated with this manuscript.

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