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Virology

Possibility of mechanical transmission of parapoxvirus by houseflies (*Musca domestica*) on cattle and sheep farms

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ABSTRACT. Parapoxvirus (PPV) causes papular stomatitis and contagious pustular dermatitis in ruminants worldwide. The virus is generally transmitted through close contact with skin lesions containing PPV in infected animals and indirectly through PPV-contaminated materials. PPV-infected animals frequently do not show clinical signs and the route of PPV transmission is sometimes unclear. In this study, the possibility of mechanical transmission of PPV by houseflies (*Musca domestica*) was investigated using polymerase chain reaction (PCR) gene surveillance. Samples were collected from cattle, sheep, barn environments, direct wash solution of the body surface of houseflies, and indirect wash solution of the body surface and feces of the flies. Bovine papular stomatitis virus, pseudocowpox virus, and orf virus were detected in the oral cavity and body surface of cattle and sheep without clinical signs of PPV infection or barn environments; PPV was considered to have been retained on the farm. PPVs were also detected in the direct wash solution of the body surface of houseflies, and the indirect wash solution of the body surface and feces of the flies. The viral sequence determined from the indirect wash solution of the body surface and feces of the flies was identical to that determined from the body surface of cattle and barns. These results suggested that houseflies may mechanically transmit PPV to both cattle and sheep.

KEYWORDS: arthropod, barn environments, housefly, mechanical transmission, parapoxvirus

Bovine papular stomatitis, pseudocowpox, and contagious pustular dermatitis are caused by infection with bovine papular stomatitis virus (BPSV), pseudocowpox virus (PCPV), and orf virus (ORFV), all of which belong to the genus *Parapoxvirus* (PPV) [7–9]. PPV is transmitted through close contact with infected skin lesions or virus-contaminated foodstuffs and materials on farms. It forms papules, nodules, and crusts mainly on the nose, lips, and oral cavity. Furthermore, it is an infectious zoonotic disease important to distinguish from foot-and-mouth disease in cattle, sheep, and goats [10].

The habitats (distribution areas) of arthropods, such as insects and mites, are expanding due to global warming. Several studies have demonstrated the expansion of habitats and spread of infectious diseases borne by blood-sucking arthropods, such as mosquitoes, which transmit Japanese encephalitis and dengue viruses [1, 5, 18, 29]. The mechanical transmission of pathogens by non-blood-sucking arthropods that occurs when these pathogens attach to body surfaces has been reported for bacteria and parasites [13]; however, the possibility of viruses being mechanically transmitted by non-blood-sucking arthropods and spreading infectious diseases has not yet been analyzed in detail, although experimental data have been reported [2, 19]. In this study, we used PPV as a model to examine the following sources of genetic material: the oral cavity and body surface of cattle and sheep, barn environments, direct wash solution of the body surface of houseflies (*Musca domestica)*, a non-blood-sucking arthropod, and indirect wash solution of the body surface and feces of flies from the inside of the plastic bag in which they were captured. Using these data, we examined the conditions caused by PPV infection and contamination on farms, and the possibility that adult houseflies can mechanically transmit PPVs.

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MATERIALS AND METHODS

Detection of PPV DNA from cattle, sheep, and barn environments

The study sites were as follows: Yanagido Farm of Gifu University, which keeps approximately 20 Holstein cattle; Cattle Farm A, which keeps approximately 40 Holstein cattle; Cattle Farm B, which keeps approximately 80 Holstein cattle; and Sheep Farm, which keeps approximately 200 Suffolk sheep. At Yanagido Farm, the barn was renovated during this research period and completed in July 2021; thus, we surveyed the old barn from February 2020 to June 2021 and the renovated barn from July 2021 to October 2021. Cattle Farm A was surveyed in February and August 2021, Cattle Farm B in December 2021, and Sheep Farm in July 2020 and May 2021. None of the cattle or sheep surveyed in this study showed clinical signs of PPV infection. The oral cavity and body surfaces of cattle and sheep and their barn environments were wiped with sterile cotton swabs and diluted with 2 mL phosphate-buffered saline (PBS) to prepare the samples for surveillance of PPV DNA by polymerase chain reaction (PCR). The diluted specimens were mixed well, and 1 mL was transferred to a microtube and centrifuged at 20,400 × *g* for 1 hr at 20–25°C. Total DNA was extracted from 200 µL of the lower layer after centrifugation using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and DNA was eluted from the column with 30 µL of the buffer AE. PCR was performed to detect the PPV gene using primers PPP-1, PPP-4, and PPP-3 [11], which amplify the PPV envelope region, and GoTaq Hot Start Green Master Mix (Promega, Madison, WI, USA) as described previously [22, 23]. PCR was carried out in a total reaction volume of 20 µL containing polymerase (0.5 U), forward and reverse primers (0.5 µM each), and extracted DNA (5 µL). Semi-nested PCR was performed using 1 µL of PCR product. The thermal cycling conditions were as follows: 2 min at 95°C, followed by 40 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 7 min. A positive result was determined by the presence of a 235 bp band of the semi-nested PCR product on agarose gel electrophoresis.

Arthropod capture materials

The five types of materials used for capturing the arthropods were used as follows: Knockbait (Elanco Japan Co., Ltd., Tokyo, Japan), 1% Agita (Elanco Japan Co., Ltd.) that mainly targeted adult houseflies, T-traps (T-Set Corp., Kariya, Japan), Mou Anshin (Sunpack Co., Ltd., Kurayoshi, Japan), and Sticky Roll (Coburn Co., Whitewater, WI, USA) (Fig. 1A–E). These materials were installed at Yanagido Farm, Gifu University, for one week from November 9 to November 16, 2020 (Sticky Roll alone was used from October 5 to October 6, 2020). The number of captured arthropods with body length ≥5.0 mm was counted, and the arthropod species were identified by morphological observation. For electron microscopy, the surfaces of the captured adult houseflies were osmium-coated using an osmium plasma coater (OPC80AJ, Filgen, Nagoya, Japan) and examined using a scanning electron microscope S-4800 (Hitachi High-Tech, Tokyo, Japan).

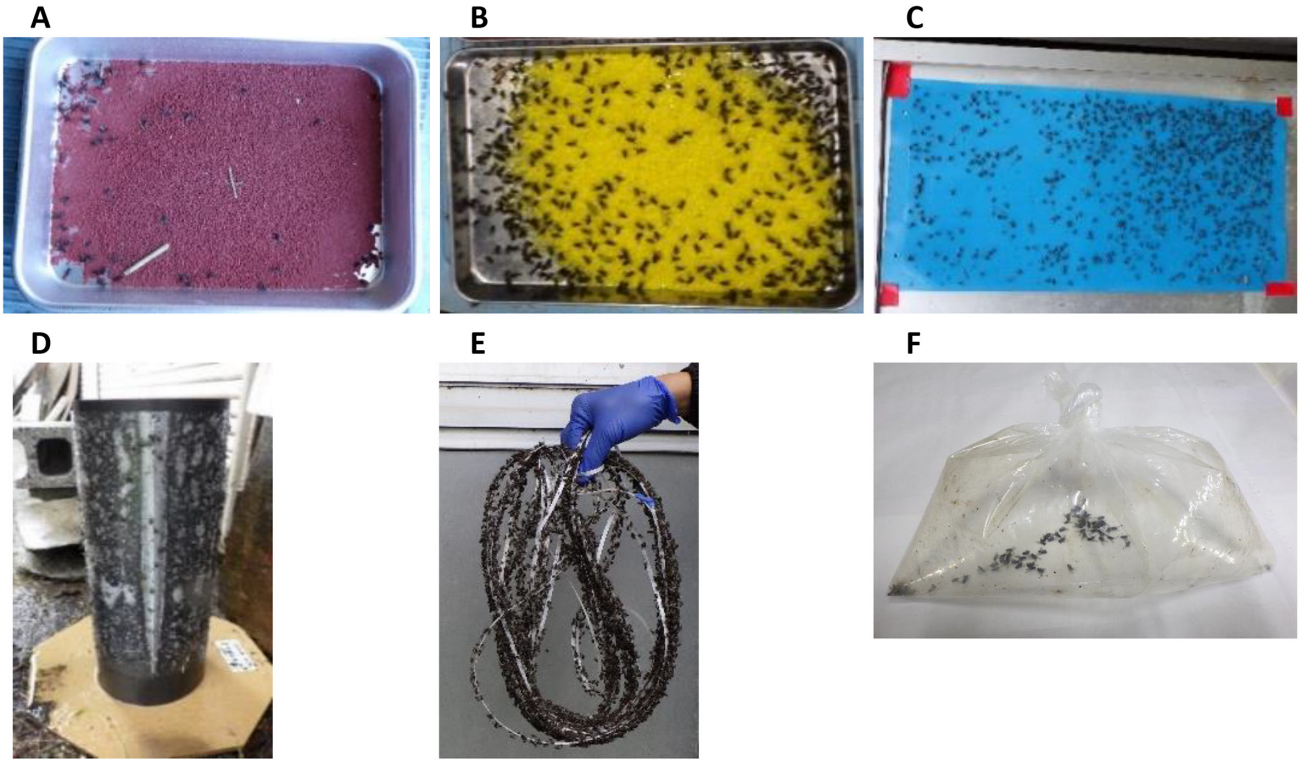

Fig. 1. Materials for capturing arthropods. Knockbait (**A**), 1% Agita (**B**), T-trap (**C**), Mou Anshin (**D**), Sticky Roll (**E**), plastic bad (**F**). The materials were installed at heights of 32 (A), 32 (B), 70 (C), 0 (D), and 80 cm (E) from the floor.

Detection of PPV DNA from flies

From November 2020 to October 2021, two types of capture materials, Knockbait and 1% Agita, were installed at Yanagido Farm, Gifu University, for 12 months (once a month for one week). The number of captured houseflies was counted, and significant differences were statistically analyzed using the Mann-Withney *U* test with a corrected *P*-value cut-off of 0.05. In addition, flies on the bodies of the animals and inside and outside the barns were captured with plastic bags using like a butterfly net (Fig. 1F). In some cases, a few stable flies (*Stomoxys calcitrans*) were accidentally captured in the bag alongside the houseflies. At Cattle Farm A, two types of capture materials were installed for only one week, from August 17 to August 24, 2021. At Cattle Farm B, flies were captured using plastic bags on October 8, 2021. After collection using materials and plastic bags, the flies were transferred to our laboratory, and precautions were taken to prevent secondary viral pollution during sample delivery or preparation. All flies collected in plastic bags were killed by placing them at −80°C and were stored at −30°C until use. The captured houseflies were collected in groups of 1–10, 11–50, or 100 houseflies in a 15 mL tube and used as one pooled sample. The body surfaces of these houseflies were washed by adding 1, 2, or 3 mL of PBS to each pooled sample and using a small rotary incubator RT-50 (Taitec, Koshigaya, Japan) to wash them without crushing or releasing their bodily fluids. One mL of the wash solution was then transferred to a microtube as a direct wash solution of the body surface of houseflies. The inside of the plastic bag used to capture flies (Fig. 1F) was washed with 6 mL of PBS, and 1 mL was transferred to a microtube as an indirect wash solution for the body surface and feces of flies. The microtubes containing 1 mL of sample were centrifuged at 20,400 × *g* for 1 hr at 20–25°C, total DNA was extracted from the lower 200 µL, and PPV DNA was detected by PCR as described above.

Molecular epidemiological analysis of PPV

The PCR products of PPV DNA detected in each sample were purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Duren, Germany). Cycle sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA). The determined sequences were used to create a molecular phylogenetic tree using MEGA7 [14] with known Japanese PPV sequence data collected from GenBank using Genetyx-Win software version 13 (Genetyx, Tokyo, Japan) and the maximum likelihood method.

RESULTS

Detection of PPV DNA from cattle, sheep, and barn environments

PPV DNA was detected in 10 out of 21 oral cavity samples from cattle and 10 out of 47 environmental samples from the old barn, but not in samples from the renovated barn of Yanagido Farm, Gifu University (Table 1). At Cattle Farm A, PPV DNA was detected in one out of 20 environmental samples in February 2021, but not in August 2021. At Cattle Farm B, PPV DNA was detected in three out of 20 cattle body surface samples and in three out of 20 environmental samples. In July 2020 at Sheep Farm, PPV DNA was detected in 19 out of 67 sheep oral cavity samples, 12 out of 67 sheep body surface samples, and four out of 20 environmental samples. In May 2021, PPV DNA was detected in 16 out of 51 sheep oral cavity samples and in six out of 51 sheep body surface samples.

Number of captured arthropods by materials

As a result of capturing arthropods using the five materials, it became clear that the captured arthropod species differed greatly depending on the material (Supplementary Table 1). Most of the arthropods captured using Mou Anshin, T-trap, and Sticky Roll were adult stable flies, which are blood-sucking arthropods. Knockbait and 1% Agita granules captured the largest number of adult houseflies. Therefore, these two materials were used for the monthly surveillance of houseflies at Yanagido Farm, Gifu University.

Electron microscopic observation of the body surface of houseflies

The body surface of the houseflies was covered with body hairs of various thicknesses and lengths, and numerous small hairs were observed on the underside of the forewings (Fig. 2A and 2B). Fine particles adhered to the small hairs and bristles on the underside of the forewings (Fig. 2C and 2D).

Table 1. Detection of parapoxvirus DNA from cattle, sheep, and barn enviroments*

Farms	Sampling periods	Oral cavity	Body surfaces	Barn environment
Yanagido Farm Gifu University	Old barn from February 2020 to June 2021	10/21 (47.6%)	0/19 (0.0%)	10/47 (21.3%)
	Renovated barn from July 2021 to October 2021	0/16 (0.0%)	0/16 (0.0%)	0/11 (0.0%)
Cattle Farm A	February 2021	-	0/20 (0.0%)	1/20 (5.0%)
	August 2021	-	0/20 (0.0%)	0/20 (0.0%)
Cattle Farm B	December 2021	-	3/20 (15.0%)	3/20 (15.0%)
Sheep Farm	July 2020	19/67 (28.4%)	12/67 (17.9%)	4/20 (20.0%)
	May 2021	16/51 (31.4%)	6/51 (11.8%)	0/19 (0.0%)

*Positive number/number of samples. A positive result was determined by the presence of a band of 235 bp by agarose gel electrophoresis.

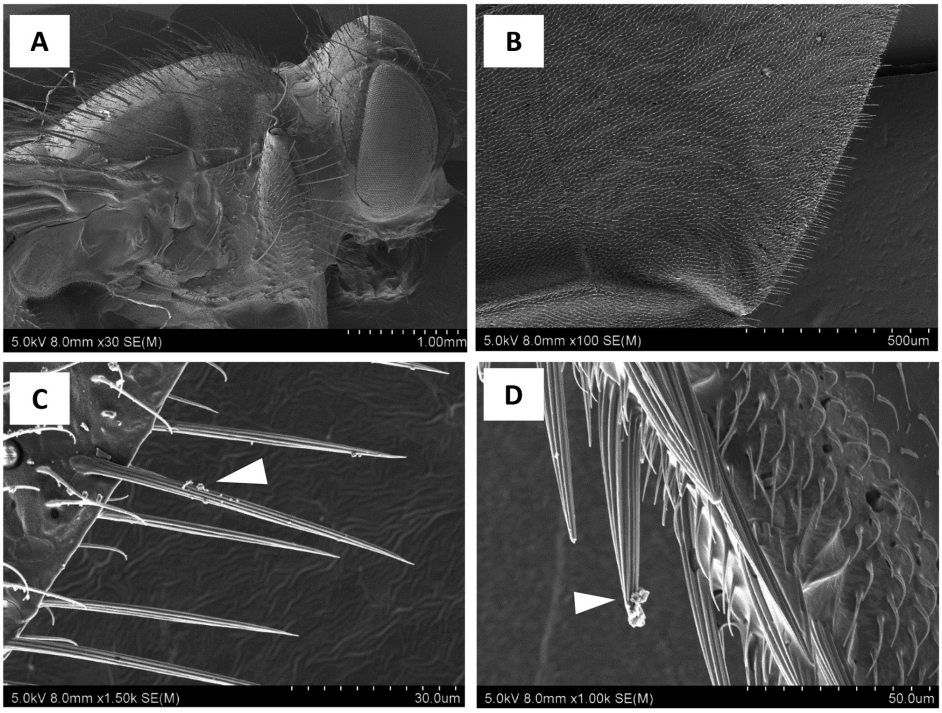

Fig. 2. Body surface of a housefly photographed by scanning electron microscopy. Head, chest (**A**), back of front wing (**B**), and enlargement of the back of front wing (**C**, **D**). Fine particles can be observed attached to the small hairs and bristles of the front wing (arrowhead).

Detection of PPV DNA from flies

During the research period, the number of captured adult houseflies at Yanagido Farm, Gifu University, from January 2021 to June 2021 was small; however, this number increased sharply from July 2021 and exceeded 1,000 for both materials in August 2021 (Fig. 3). At Cattle Farm A, five houseflies were captured in August. Each material (Knockbait and 1% Agita) differed in the attractant, color, and shape preferred by houseflies; however, no significant differences were observed in the number of captured houseflies (P=0.717). Furthermore, even from June to December, when the number of captured houseflies per week at Yanagido Farm was as low as ≤50, the number of captured arthropods with a body length of <5.0 mm was constant (Supplementary Fig. 1). From November 2020 to October 2021 at Yanagido Farm, 1,286 and 450 houseflies captured by the materials and plastic bags, respectively, were used to detect PPV DNA from direct wash solution samples. However, all results were negative for PPV DNA (Table 2). All indirect wash solutions/feces samples were also negative. At Cattle Farm A, a direct wash solution sample from five houseflies captured by the materials (Knockbait and 1% Agita) was negative. At Cattle Farm B, PPV DNA was detected in two out of three samples of the direct wash solution from 50 captured houseflies by plastic bags and one out of two samples of the indirect wash solution/feces of flies.

Molecular epidemiological analysis of PPV

Nucleotide sequences were determined using PCR products obtained from four out of 20 positive samples from Yanagido Farm, Gifu University, one out of one positive sample from Cattle Farm A, eight out of nine positive samples from Cattle Farm B, and 38 out of 57 positive samples from Sheep Farm. Some PCR products cannot be sequenced because of their low DNA concentration or purity. All DNAs detected in the old barn of Yanagido Farm had the same sequence and belonged to the BPSV cluster in the phylogenetic tree (Fig. 4). The DNA detected in the barn environment of Cattle Farm A belonged to the BPSV cluster. In Cattle Farm B, the DNAs detected in the cattle body surface, barn environment, and indirect wash solution/feces of flies had the same sequence and belonged to the same BPSV cluster, indicating the possibility of mechanical transmission of BPSV by flies. However, the DNA detected in the direct wash solution of the houseflies belonged to the PCPV cluster. The DNAs detected at Sheep Farm in 2020 belonged to the BPSV, PCPV, and ORFV clusters. In addition, PCPV clusters were divided into two clusters. The DNAs detected at Sheep Farm in 2021 belonged to the cluster of PCPV and ORFV, but differed from the cluster of the DNAs detected in 2020.

DISCUSSION

In this study, PPV DNAs were detected in the direct wash solution of the body surface of houseflies and in the indirect wash solution of the body surface and feces of flies. The BPSV detected in the indirect wash solution possessed the same sequence as that detected in the cattle body surface and barn environments. Viral genes are detected at a high rate on the body surface and in the feces of houseflies experimentally exposed to SARS-CoV-2 [2] and ORFV [19], and viral genes are detected on the body surface of houseflies in areas

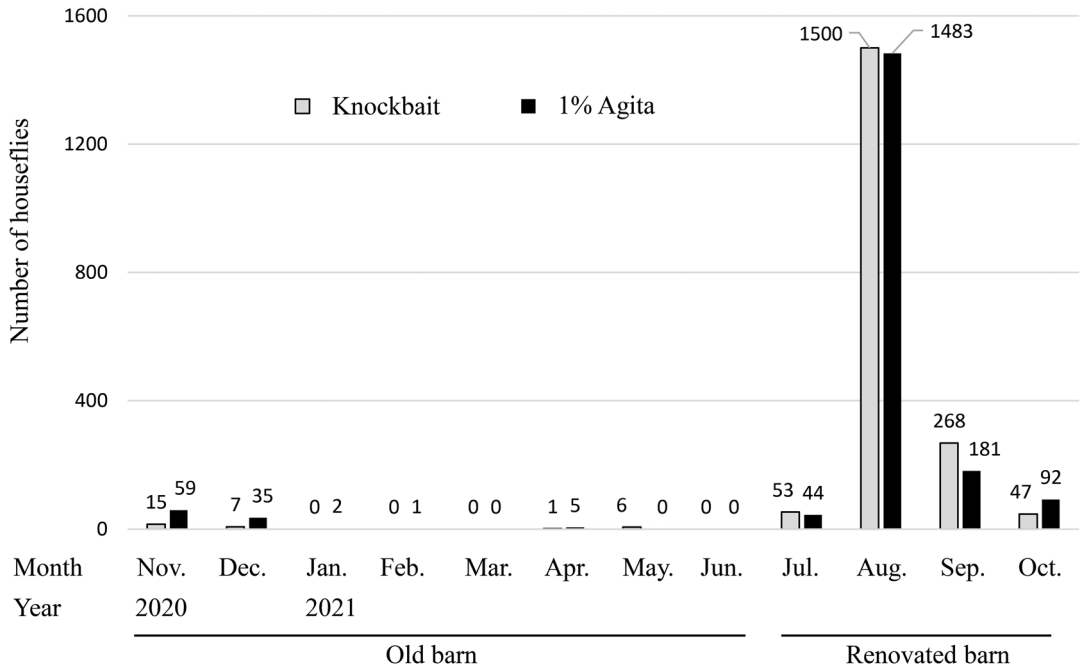

Fig. 3. Number of captured houseflies per month at Yanagido Farm, Gifu University. Two types of materials (Knockbait and 1% Agita) were installed in the barn once a month for one week, and the number of captured houseflies was counted.

Table 2. Detection of parapoxvirus DNA from flies*

		Yanagido Farm Gifu University	Cattle Farm A	Cattle Farm B
Knockbait	Direct wash solution of houseflies	0/24 (627)	0/1 (5)	-
1% Agita	Direct wash solution of houseflies	0/29 (659)		-
Plastic bag	Direct wash solution of houseflies	0/20 (450)	-	2/3 (50)
	Indirect wash solution/feces of flies	0/17	-	1/2

*Positive number/number of pooled samples (total houseflies); direct wash solution of houseflies, the body surfaces of pooled houseflies were washed without crushing or releasing their bodily fluids; indirect wash solution/feces, the inside of the plastic bag used to capture flies was washed with phosphate-buffered saline. A positive result was determined by the presence of a band of 235 bp by agarose gel electrophoresis.

where SARS-CoV-2 [24] and lumpy skin disease virus [25] infections are endemic. In addition, in this study, multiple particles attached to the body surface of captured houseflies were observed by scanning electron microscopy. These findings suggest that houseflies are structurally capable of picking up and mechanically carrying PPV by utilizing their adhesive-covered legs and fine hair-covered wings, as has been reported for bacteria and fungi [13]. Houseflies have a flying range of 5–7 km [17], suggesting that they may be able to spread PPVs a large distance. However, rotaviruses [27] attached to housefly legs and wings are shed prematurely due to their behavior. Despite houseflies carrying SARS-CoV-2 [2], Ebola virus [6], and Newcastle disease virus [28] for long periods, they have insufficient amounts to cause infection. The possibility of mechanical transmission of PPV by houseflies should be further examined by measuring the amount of virus detected on the body surface of houseflies, and by examining the infectivity of the detected virus and determining whether the amount of virus is sufficient for successful pathogenesis.

BPSV, PCPV, and ORFV DNAs were detected in the oral cavity and on the body surface of cattle and sheep that did not show clinical signs of PPV infection, as well as in barn environments. This suggests that the virus was retained in individual livestock and barn environments. PPV has been reported to exist in the environment for a long time, and reinfection can occur [3, 7–10, 16], suggesting a risk of PPV infection at Cattle Farm B and Sheep Farm. PPV genes were not detected in the second survey of Yanagido Farm and Cattle Farm A. Yanagido Farm had recently undergone renovation, and only a small number of viruses were detected at Cattle Farm A in the first survey. PPV genes were considered undetectable due to the low viral concentration in these barns. In addition, at Yanagido Farm, the capture method and the number of houseflies to be pooled were examined; however, the PPV gene was not detected in any of the samples. From these results, it was concluded that the PPV concentration in the direct wash solution of the body surface of houseflies was less than that of the livestock and barn environment samples, and that the houseflies were not contaminated with PPV because the PPV concentration in the renovated barn was low. Previously, real-time PCR was used for PPV detection in animals

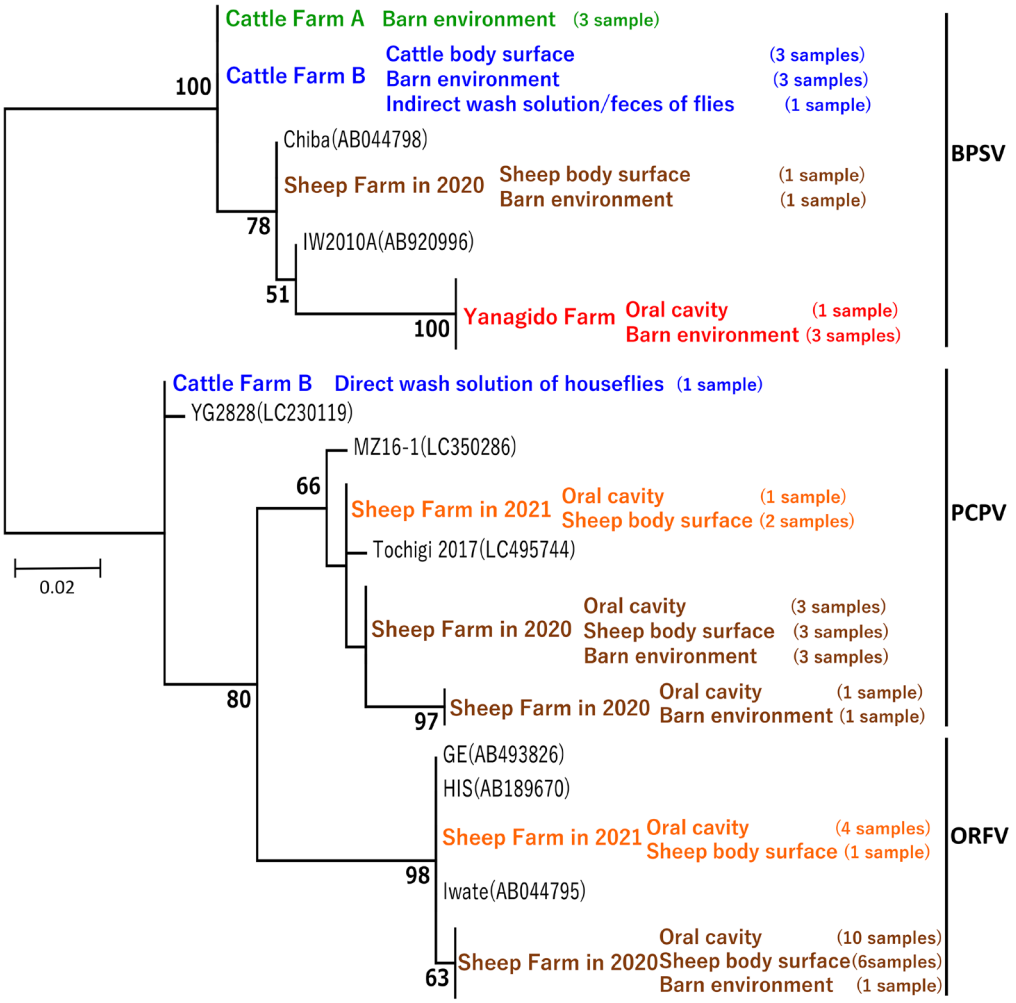

Fig. 4. Molecular phylogenetic tree of the envelope region (215 bp) sequences of parapoxviruses in Japan. The numerical value of the phylogenetic tree indicates the bootstrap value (1,000 operations). Scale bar inducates the base substitution rate. All known Japanese strains are listed in the order of their strain names (GenBank accession number). The virus species are shown on the right-hand side of the phylogenetic tree. Parapoxvirus detected at Yanagido Farm, Cattle Farm A, Cattle Farm B, Sheep Farm in 2020, and Sheep Farm in 2021 are shown as red, green, blue, brown, and orange, respectively. BPSV, bovine papular stomatitis virus; PCPV, pseudocowpox virus; ORFV, orf virus; direct wash solution of houseflies, the body surfaces of pooled houseflies were washed without crushing or releasing their bodily fluids; indirect wash solution/feces of flies, the inside of the plastic bag used to capture flies was washed with phosphate-buffered saline.

without clinical signs [4, 15, 20, 30], in barn environments [15, 30], and on the body surface of houseflies [20]. Friederichs *et al.* [4] reported that some samples from animals without clinical signs were positive by real-time PCR but negative by conventional PCR. Furthermore, Yaegashi *et al.* [30] showed that PPV DNA copy numbers are significantly lower in animals without clinical signs. These results suggest that the conventional PCR method used in this study has lower detection ability than real-time PCR.

Cattle were the main hosts of BPSV and PCPV, whereas sheep and goats were the main hosts of ORFV. Our previous studies revealed the genetic diversity of PPV among different hosts [22, 23, 26]. However, in the present study, BPSV and PCPV were detected in the oral cavity and on the body surface, respectively, of sheep, which are not the main hosts. The Sheep Farm is located in a mountainous area where sheep are grazed and wild animals such as Japanese serows (*Capricornis crispus*) are present. Moreover, some employees and veterinarians working on the Sheep Farm are involved in cattle breeding. Based on the aforementioned facts, it is proposed that a viral gene from outside the host range was detected. Previous antibody tests have revealed that a large percentage of cattle [21] and wild Japanese serows [12] in Japan are infected with PPV. To evaluate PPV contamination in barns, it is necessary to consider contamination due to PPV release from positive animals and carry out antibody tests in parallel.

In conclusion, this study indicates that houseflies may be capable of mechanically transmitting PPV and that PPV is preserved in individual livestock and barn environments. Arthropod control measures are necessary to prevent the spread of PPV, Further studies are required to verify the possibility of mechanical transmission of the virus by arthropods, including non-blood-sucking houseflies.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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