



NOTE

Virology

Sequential detection of pseudocowpox virus and bovine papular stomatitis virus in a same calf in Japan

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ABSTRACT. We detected parapoxviruses from environmental samples and calves with and without intraoral clinical signs and conducted molecular and serological analyses. Pseudocowpox virus (PCPV) was detected from a calf showing anorexia, frothy salivation, and erosion in the mucosa of the lip and tongue. At the time that PCPV was detected, bovine papular stomatitis viruses (BPSVs) were detected in environmental samples as well as in calves without intraoral clinical signs. BPSV, but not PCPV, was detected in the same calf after 22 days. Phylogenetic analysis revealed that genetically different PCPV strains exist in Japan. This is the first report on the detection of PCPV and BPSV sequentially in the same calf and coexistence of PCPV and BPSV in the same farm in Japan.

KEY WORDS: bovine papular stomatitis virus, calf, coexistence, pseudocowpox virus

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The genus *Parapoxvirus*, in the family *Poxviridae*, includes four members: bovine papular stomatitis virus (BPSV), pseudocowpox virus (PCPV), orf virus (ORFV), and parapoxvirus of red deer in New Zealand (PVNZ) [12]. Parapoxvirus infections are widespread in ruminants worldwide and usually cause mild papules, erosions, and pustules in the skin around the muzzle, mouth, and teats [2, 12]. It is very important to differentiate these infections from serious infectious diseases such as foot-and-mouth disease in livestock industry as the clinical signs are similar [1, 3].

In Japan, a serological survey of parapoxvirus infection among cattle indicated very high positivity rates (between 40 and 98%) [14], and several cases of BPSV infections have been reported previously from cattle [7, 9, 16]. BPSV can cause subclinical infection, reinfection, and persistent infection in cattle [5, 13, 17]. Only two cases of PCPV infection have been reported previously in Japan [11, 16], and therefore epidemiological status of PCPV infection in cattle in Japan remains unclear.

In this study, we report a parapoxvirus infection in Miyazaki Prefecture, Japan, in August 2017. PCPV was detected from an oral swab sample of a calf with intraoral clinical signs. Interestingly, BPSV, but not PCPV, was detected in the same calf after 22 days. We determined the nucleotide sequence of the PCPV strain and performed phylogenetic analysis.

In August 2017, a calf (crossbred cattle, male, 6-month old, cattle ID A-1) on a farm (farm A: fattening farm) in Miyazaki Prefecture, Japan, showed anorexia, frothy salivation, and erosion in the mucosa of the lip and tongue. Three other calves (cattle ID A-2 to A-4) housed in the same pen showed no clinical signs. All calves (cattle A-1 to A-4) had just been introduced from Hokkaido, Japan, three weeks before the appearance of intraoral clinical signs. On this farm, calves were usually introduced regularly from Hokkaido, Chiba and Okinawa Prefectures, Japan. The location of the cattle is shown in Fig. 1.

Virological analysis was carried out using oral swab samples collected from the mucosal lesions of a calf (cattle A-1), at the time of appearance of intraoral clinical signs (Table 1). At the same time, oral swab samples were collected from calves without intraoral clinical signs (cattle A-2 to A-4) housed in the same pen, which are indicated as “pre samples” in Table 1, and environmental samples from the fences, feeding containers, and water cups around the affected calf were also collected (Table

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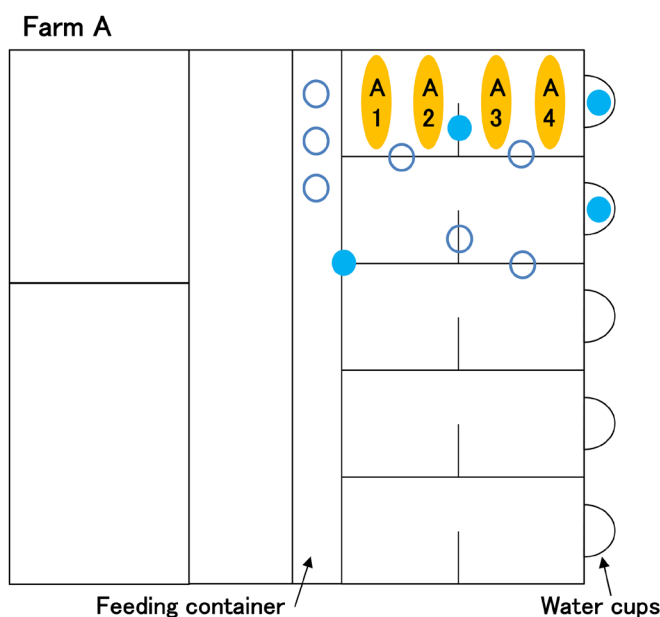


Fig. 1. Location of calves and sampling of environmental swabs. Filled circles, PCR-positive. Open circles, PCR-negative.

Table 1. Summary of the virological and serological analyses for parapoxvirus infection

Farm	Cattle ID	Breed	Sex	Age (month)	Intraoral clinical signs	PCR ^{a)}				AGID test ^{b)}			
						Pre ^{c)}	(RFLP)	Strain	Accession no.	Post ^{c)}	(RFLP)	Pre ^{c)}	Post ^{c)}
A	A-1	Crossbred	Male	6	Anorexia, frothy salivation, and erosion in the mucosa of the lip and tongue	+(Aug. 29)	(PCPV)	MZ17-3	LC350284	+(Sep. 20)	(BPSV)	+(Aug. 29)	+(Sep. 20)
	A-2	Crossbred	Male	7	-	+(Aug. 29)	(BPSV)			+(Sep. 20)	(BPSV)	ND	ND
	A-3	Crossbred	Male	6	-	+(Aug. 29)	(BPSV)	MZ17-4	LC350285	+(Sep. 20)	(BPSV)	+(Aug. 29)	+(Sep. 20)
	A-4	Crossbred	Male	6	-	+(Aug. 29)	(BPSV)			-(Sep. 20)		+(Aug. 29)	+(Sep. 20)
B	B-1	Holstein	Female	39	-	+(Jul. 29)	(PCPV)	MZ16-1	LC350286	ND		ND	ND

a) RFLP, restriction fragment length polymorphism; BPSV, bovine papular stomatitis virus-pattern; PCPV, pseudocowpox virus-pattern; ND, not determined.
b) AGID, agar gel immunodiffusion; ND, not determined. c) Parenthesis indicates sampling date.

1 and Fig. 1). After 22 days, oral swab samples were collected from all calves again, which are indicated as “post samples” in Table 1. The swab samples were homogenized with phosphate buffered saline (PBS) and centrifuged at $1,270 \times g$ for 5 min at 4°C. The supernatants were filtered through a 450-nm pore sized membrane (Merck Millipore, Cork, Ireland). For virus isolation, swab samples were inoculated into primary bovine testis (BT) cells in rolling tubes by rotary cultures as described previously [11]. Cells were passaged more than three times in a blind manner. Viral DNA was extracted from swab samples with a High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany). Polymerase chain reaction (PCR) amplifications were carried out with a Taq PCR Master Mix Kit (Qiagen, Hilden, Germany) using a TaKaRa Thermal Cycler Dice Touch (TaKaRa Bio, Kusatsu, Japan) and the primer set PPP-1/PPP-4 for detection of the partial-length (594bp) *B2L* gene, which encodes the parapoxvirus envelope [6]. Restriction fragment length polymorphism (RFLP) analysis was conducted with *Drd* I (marker for ORFV), *Xmn* I (marker for BPSV), and *PfM* I (marker for PCPV) as described previously [7]. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and the nucleotide sequence was determined by direct sequencing using a BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Austin, TX, U.S.A.). Sequence data was aligned using the ClustalW method [15] and phylogenetic analysis was performed using MEGA7 software [8]. Phylogenetic trees were constructed using maximum-likelihood estimation methods, and the reliability of the branches was evaluated by bootstrapping with 1,000 replicates. The nucleotide and deduced amino acid sequences were compared with those of available corresponding parapoxviruses.

For serological analysis, serum samples were collected from all calves at the time of appearance of intraoral clinical signs and after 22 days again (Table 1) and used for agar gel immunodiffusion (AGID) tests as described previously [9]. Oral swab sampling and blood collection were performed within the veterinary scope of practice with informed owner consent. This study was approved by the Gifu University Animal Care and Use Committee (Approval numbers 14094 and 17046).

No cytopathic effects were observed in BT cells, and thus viral isolation was unsuccessful. PCR specific for the partial

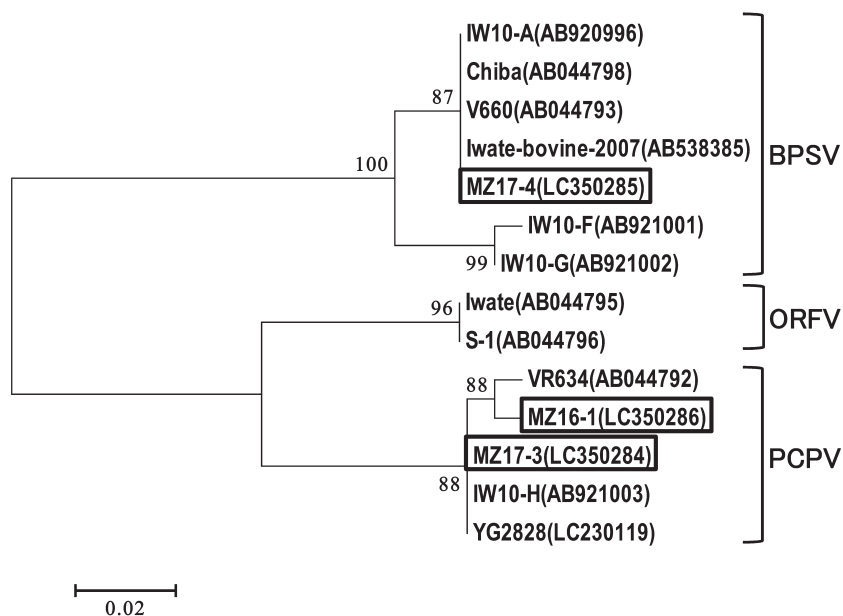


Fig. 2. Phylogenetic tree based on the deduced amino acid sequences of the partial *B2L* gene of parapoxviruses. The percentage bootstrap values calculated from 1,000 replicates are indicated above the internal nodes. Three strains detected in this study are shown in boxes.

parapoxvirus *B2L* gene showed positive results from all the oral swab samples of the first collection (pre samples) and from three of the oral swab samples of the second collection (post samples) (Table 1). By RFLP analysis, three PCR products from the oral swab samples of the first collection (pre samples from cattle A-2 to A-4) and three PCR products from the oral swab samples of the second collection (post samples from cattle A-1 to A-3) were cut with *Xmn* I and thus classified as BPSV; however, a product of the oral swab sample of the first collection (pre sample) from A-1 was cut with *Pfl*M I and was therefore classified as PCPV (Table 1). The nucleotide sequences of two representative PCR products which were amplified clearly were determined and designated as MZ17-3 (cattle A-1, GenBank/EMBL/DBJ accession no. LC350284) and MZ17-4 (A-3, LC350285). Based on the nucleotide/deduced amino acid identities and phylogenetic analysis, the MZ17-4 strain was classified as BPSV; however, the MZ17-3 strain was classified as PCPV in accordance with the RFLP analysis. The phylogenetic tree showed that MZ17-4 clustered with the previously reported BPSV strains Iwate-bovine-2007 (AB538385) and Chiba (AB044798), which were isolated from cattle in Iwate and Chiba Prefectures, Japan, and with V660 (AB044793), which was isolated from a calf in Europe. MZ17-3 clustered with the previously reported PCPV strains YG2828 (LC230119) and IW10-H (AB921003), which were isolated from cattle in Yamaguchi and Iwate Prefectures. Additionally, a nucleotide sequence was determined from a stored DNA sample which was previously extracted from the oral cavity of a cow (Holstein, female, 39-month old, cattle ID B-1) without intraoral clinical signs on a farm (farm B: which is located south of farm A and is about 70 km away, and there is no epidemiological relation with farm A) in Miyazaki Prefecture in 2016, and the PCPV pattern on RFLP analysis was observed and designated as MZ16-1 (LC350286) (Table 1). MZ16-1 was classified as PCPV, but instead of clustering with the same group as MZ17-3, it clustered with another group of PCPV, the VR634 strain (AB044792), which was isolated from a human in the United States of America (Fig. 2).

In the environmental samples, PCR showed positive results from four samples out of 11 at farm A (Fig. 1). RFLP analysis using PCR products selected from farm A revealed that all of them were cut with *Xmn* I (data not shown) and thus classified as BPSV.

The AGID test showed positive results from both serum samples of the first (pre samples) and second (post samples) collection (Table 1).

The detection of multiple parapoxviruses in cattle has been reported overseas [4, 10], but not in Japan. Moreover, coexistence of multiple parapoxvirus members on a farm has not been reported in Japan. In this study, PCPV and BPSV were detected sequentially from the same calf with intraoral clinical signs; however, although BPSVs were detected, no PCPV was simultaneously detected in other calves or the environment. This is the first report of PCPV and BPSV detection in the same calf sequentially. It is also the first report to describe the coexistence of PCPV and BPSV at the same time on a farm in Japan. As BPSV was detected in calves without clinical signs and the environment, we speculated that the calf (A-1) may be already infected with PCPV subclinically in Hokkaido and showed intraoral clinical signs after transport to the farm A, and that BPSV may exist in calves subclinically (except the calf (A-1)) and in the environment of the farm A. Presumably, the fact that calves on farm A are regularly introduced from three regions may be a possible reason of the coexistence of multiple parapoxvirus members. The clinical signs seen in the calf infected with PCPV were similar to clinical signs reported in a previous study of PCPV infection in Japan, such as anorexia, frothy salivation, and hyperemia in the mucosa under the tongue surface [11].

BPSV was detected in all calves; two calves (A-2 and A-3) had positive oral swab samples from the first and second collection, BPSV was detected in environmental samples as well. Additionally, before the appearance of intraoral clinical signs, calves had

just been introduced from Hokkaido. Previous studies reported that BPSV caused subclinical infection, persistent infection, and reinfection [5, 13, 17], and that parapoxvirus infection can be induced by stress factors [13, 17]. Therefore, we hypothesize that parapoxvirus infection in this study may be related to transport. BPSV could have caused subclinical infection and incubated for a long period in the calf oral cavity; then, BPSV-infected calves could have exhaled the virus and contaminated the farm environment in consideration of the resistance of BPSV in nature.

In this study, two genetically different strains of PCPV (MZ16-1 and MZ17-3) were detected in Miyazaki Prefecture. MZ17-3, which was detected at farm A, clustered with two PCPV strains previously reported in Japan (IW10-H [16] and YG2828 [11]). These are the only two PCPV strains reported in Japan and were detected from cattle showing papular stomatitis, but not clinical signs on the teats and udder. However, MZ16-1, which was detected from a calf without intraoral clinical signs at farm B in 2016, was in a different cluster from the PCPV IW10-H and YG2828 strains. These results suggest that multiple genetically different PCPV strains are spreading among cattle with or without intraoral clinical signs in Japan.

It has been reported overseas, that co-infection with multiple BPSVs causes more severe clinical signs [4]. In Japan, only two studies detecting PCPVs have been reported [11, 16], and the prevalence of coinfection of PCPV with BPSV and other viruses is unknown. Further investigations in the field and with regard to the pathology of parapoxvirus infections are needed.

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