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Molecular phylogenetic analysis of bovine papular stomatitis viruses detected in Saga, Japan

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ABSTRACT. In this study, we performed a molecular phylogenetic analysis of six bovine papular stomatitis virus (BPSV) field strains detected from Japanese beef calves kept on a farm in Saga prefecture, a southwest part of Japan, from 2017 to 2020. The phylogenetic analysis based on a partial B2L gene (554-nt) showed that these field strains were divided into two lineages, a lineage (A-lineage) constructed by a Saga strain and strains obtained from various regions of Japan and the world, and other lineage (B-lineage) constructed by five Saga strains and strains obtained from France, USA and Iwate prefecture (a north part of Japan). Furthermore, a Saga field strain named BPSV_SAGAbv2 and strains obtained from USA and Iwate prefecture belonged to a sub-lineage blanched from B-lineage. This is the first report elucidating molecular epidemiological characters of field BPSVs obtained from Saga prefecture. The existence of the multiple lineages was thought to be related to a history of calf introduction from various regions of Japan into Saga prefecture.

KEY WORDS: bovine papular stomatitis virus, histopathology, Japan, molecular phylogenetic analysis, Saga prefecture

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Bovine papular stomatitis virus (BPSV) belonging to the family *Poxviridae*, genus *Parapoxvirus*, is the etiological agent of papular, node and/or ulcer lesions on the muzzle, lips and oral mucosa of calves, and causes the zoonotic disease known as "Milker's node" [6]. This disease is rarely clinically significant and usually found incidentally in calves with different clinical problems [1]. However, it is necessary to differentiate bovine papular stomatitis from other more serious infectious diseases that cause similar gross lesions, such as foot-and-mouth disease (FMD), bovine viral diarrhea-mucosal disease (BVD-MD) and the alimentary form of infectious bovine rhinotracheitis (IBR), among others [27].

Papular stomatitis in cattle is caused not only by BPSV, but also by Pseudocowpox virus (PCPV). Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) methods, the gold standard diagnostic method in Japanese local government diagnostic centers, allow the detection and distinguishing of BPSV and PCPV, as well as orf virus (ORFV) and parapoxvirus of red deer in New Zealand (PVNZ), which belong to the same genus and form similar lesions in ruminants other than cattle [13, 14]. As sequencing methods have become widespread, molecular phylogenetic analyses of BPSV, based on the B2L gene, the most analyzed and registered gene of this virus, have been performed to compare field BPSVs strains obtained from various parts of the world; and these studies have been followed by molecular epidemiological characterizations [2, 6, 11, 18, 20, 21, 23]. Moreover, a molecular phylogenetic analysis of strains obtained in the northeast region of Japan has been reported [29]. Although, Kyushu Island that located in the southwestern part of Japan is a major Japanese beef cattle breeding area possessing 36.5% of all the cattle, the molecular epidemiology of BPSV in this island is poorly understood, that only one strain isolated in Miyazaki Prefecture has been phylogenetically analyzed [20]. Furthermore, BPSVs obtained from Saga prefecture have not been analyzed.

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In this study, we determined the 554-nt partial sequences of B2L gene, the most registered sequence of this virus, obtained from six BPSV field strains collected in Saga prefecture (a region of Kyushu), from 2017 to 2020, and analyzed these strains by using molecular phylogenetic method based on the 554-nt partial sequences of B2L gene with strains obtained from various parts of Japan and the world to elucidated its molecular epidemiological characteristics.

Oral swabs or a hard palate tissue were collected from five healthy calves and a calf died from pneumonia with incidental papular lesions, respectively. These calves were Japanese beef cattle bred in a farm in Saga prefecture from 2017 to 2020 (Table 1).

The oral swabs and tissue specimens were homogenized mechanically in Eagle's Minimum Essential Medium in a tube using a bead-beating method. Total nucleic acids were extracted from the homogenates using a Maxwell RSC Viral Total Nucleic Acid Purification Kit (Promega, Madison, WI, USA) with a Maxwell RSC Instrument (Promega), according to the manufacturer's instructions. PCR was performed with AmpliTaq Gold (Applied Biosystems, Waltham, MA, USA) using a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) and the PPP-1/PPP-4 primer set for amplification of a 594 bp region of the B2L gene, which encodes the parapoxvirus envelope [13]. Partial B2L sequences were determined by Eurofins Genomics (USA) using purified DNA amplicons. Multiple nucleotide sequence alignments of the partial B2L gene were generated with the ClustalW program and comparisons of individual nucleotide sequence identities among the SAGA BPSV field strains were performed using BioEdit software [9].

A phylogenetic tree using the multiple alignments was constructed using the neighbor joining (NJ) method with bootstrap

Table 1. Samples for genetic comparison and phylogenetic analyses

Strain name	Year	Host	Country	Prefecture	GenBank accession No.	Reference
BPSV_SAGAbv1	2017	Cattle	Japan	Saga	LC589443	Current study
BPSV_SAGAbv2	2018	Cattle	Japan	Saga	LC589444	Current study
BPSV_SAGAbv3	2018	Cattle	Japan	Saga	LC589445	Current study
BPSV_SAGAbv4	2018	Cattle	Japan	Saga	LC589446	Current study
BPSV_SAGAbv5	2018	Cattle	Japan	Saga	LC589447	Current study
BPSV_SAGAbv6	2020	Cattle	Japan	Saga	LC589448	Current study
Aomori	-N/A-	Unknown	Japan	Aomori	AB044797	[14]
Iwate/bovine/2007	2007	Cattle	Japan	Iwate	AB538385	[26]
IW2010A	2010	Cattle	Japan	Iwate	AB920996	[29]
IW2010B	2010	Cattle	Japan	Iwate	AB920997	[29]
IW2010C	2010	Cattle	Japan	Iwate	AB920998	[29]
IW2010D	2010	Cattle	Japan	Iwate	AB920999	[29]
IW2010E	2010	Cattle	Japan	Iwate	AB921000	[29]
IW2010F	2010	Cattle	Japan	Iwate	AB921001	[29]
IW2010G	2010	Cattle	Japan	Iwate	AB921002	[29]
Chiba	-N/A-	Unknown	Japan	Chiba	AB044798	[14]
Ishikawa-B	-N/A-	Cattle	Japan	Ishikawa	AB044800	[14]
Ishikawa-S	-N/A-	Serow	Japan	Ishikawa	AB044801	[14]
Nagano2019	2019	Cattle	Japan	Nagano	LC487905	[16]
BN15	2015	Cattle	Japan	Nara	LC032028	[7]
MZ17-4	2017	Cattle	Japan	Miyazaki	LC350285	[20]
9108	2009	Cattle	France		JN162119	[4]
07005	2007	Unknown	Bangladesh		GQ902054	[19]
BPSV01-TR-Elzg-2017	2017	Cattle	Turkey		MN887533	[8]
8837	2012	Cattle	South Korea		JX968998	[23]
8838	2012	Cattle	South Korea		JX968999	[23]
8839	2012	Cattle	South Korea		JX969000	[23]
Salta	2018	Cattle	Argentina		MK285566	[21]
SV716-12	2012	Cattle	Brazil		KC896639	[3]
SV819/10	2010	Cattle	Brazil		JN629089	[6]
BPS1211CHB	2011	Cattle	Mexico		KJ137716	[28]
BV-TX09c1	2009	Cattle	USA		KM875472	[12]
BV-AR02	-N/A-	Cattle	USA		AY386265	[5]
VA09186	2009	Cattle	USA		KF830860	[18]
VA0982	2009	Cattle	USA		KF830859	[18]
BPS0510CAB	2010	Cattle	USA		KJ137717	[28]
PVNZ_HL953	2013	Cervus elaphus	Germany		NC 025963	Unpublished
ORFV/2009/Korea	2009	Goat	South Korea		GQ328006	[22]
PCPV_MZ17-3	2017	Cattle	Japan	Miyazaki	LC350284	[20]

analysis (1,000 psuedoreplicates) under the Tamura 3 parameter with gamma distribution (shape parameter=0.5) model chosen by the Find Best DNA/Protein Models (ML) program calculating Bayesian Information Criterions [17], and ORFV, PCPV and PVNZ (Accession number: GQ328006, LC350284 and NC_025963, respectively) are used as an outgroup. Phylogenetic clustering supported by bootstrap values exceeding 70% was considered to indicate phylogenetic association [10]. Results were validated by the maximum likelihood (ML) method. In order to confirm the shape of the NJ tree, an ML tree was constructed with bootstrap analysis (1,000 psuedoreplicates) according to the above substitution model. All steps in this phylogenetic analysis were conducted using MEGA X software [17].

Necropsy was performed on a calf that died from pneumonia in 2020. The hard palate tissue containing the incidental papular lesion obtained by the necropsy was fixed in 10% neutral buffered formalin and embedded in paraffin (FFPE). The FFPE was sliced into 2 µm to 4 µm sections, and the sections were stained with hematoxylin and eosin (HE) or subjected to immunohistochemistry (IHC) for histological examinations. IHC staining was performed using a commercially available test kit (Histofine Simple Stain MAX-PO Multi; Nichirei Bioscience Inc., Tokyo, Japan) and anti-BPSV (Chiba strain) rabbit serum as the primary antibody serum, and hematoxylin was used for counter staining. The anti-BPSV rabbit serum was a gift provided by Dr. Yasuo Inoshima of Gifu University, and was used as an anti-parapoxvirus antibody because it cross-reacts with other parapoxviruses in addition to BPSV [15]. Small blocks taken from the 10% neutral formalin-fixed tissue were post-fixed in 1% osmium tetroxide, embedded in epoxy resin, sectioned (approximately 60 nm thick) and stained with uranyl acetate and lead citrate. The sections were then examined with a JEM-1400 Flash transmission electron microscope (JEOL, Tokyo, Japan).

A 554-nt partial B2L sequence was determined and registered to the DNA Data Bank of Japan database (Table 1). Nucleotide sequence identities were 96.5% to 100% among the six Saga field strains. BPSV_SAGAbv6 and IW2010D, and BPSV_SAGAbv1, BPSV_SAGAbv3 to 5 and IW2010G strains exhibit 100% nucleotide identity, respectively.

The 36 BPSV strains were divided into A- or B-lineages with respective 81% and 99% bootstrap values (Fig. 1). Furthermore, the A-lineage included A1- and A2-sub-lineages with 99% and 84% bootstrap values, respectively. The A-lineage contained BPSV_SAGAbv6 and the strains from various regions of Japan including Miyazaki prefecture, one area of the Kyushu Island, and strains from South Korea, Turkey, Bangladish, USA, Mexico, Brazil and Argentina. The A1-sub-lineage was a monophyletic tree composed of South Korean strains. Furthermore, the Miyazaki strain belonged to the A2-sub-lineage, which included Japanese strains obtained in Iwate, Nagano and Nara prefectures and an Argentina strain. In contrast, the B-lineage consisted of BPSV_SAGAbv1 to 5 and the France and USA strains. In addition, BPSV_SAGAbv2 was classified into the B1-sub-lineage with the strains obtained from USA and Iwate prefecture (located in the northeast region of Japan), with a significant 88% bootstrap value. Branches of the A- and B-lineages were concentrate in each terminal of the phylogenetic tree among short genetic distances.

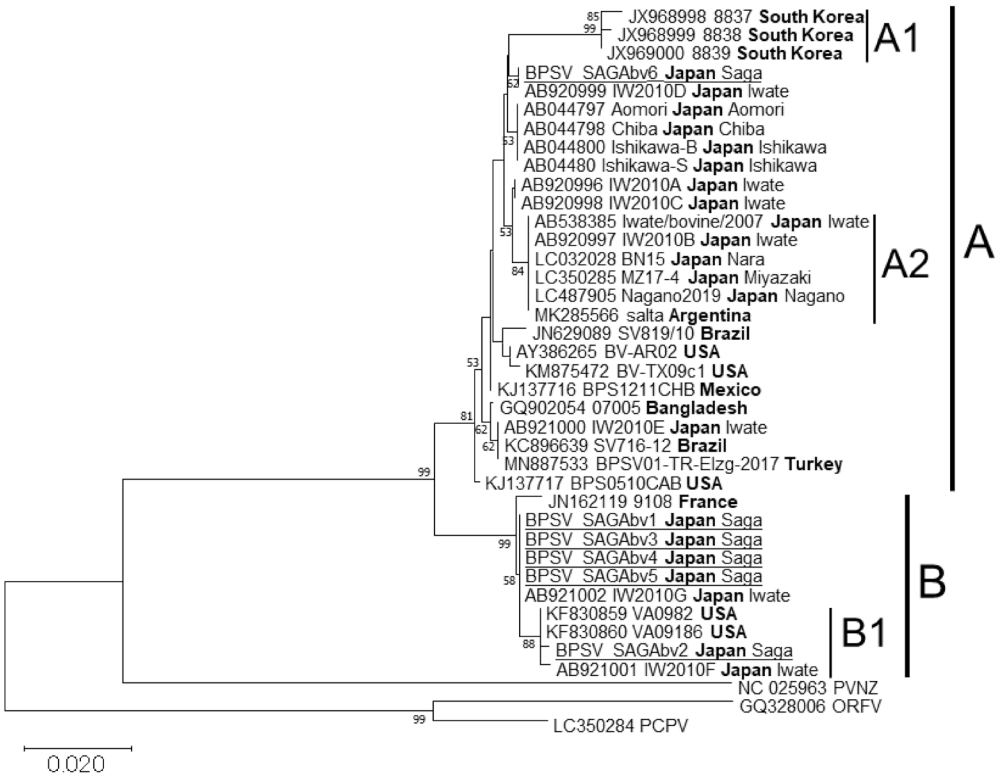

Fig. 1. A neighbor joining tree based on 554-nt partial sequence of the B2L gene. Bootstrap values above 40% are shown. Bold text indicates country names followed by prefecture names. Underlined strains were the field strains assessed in this study.

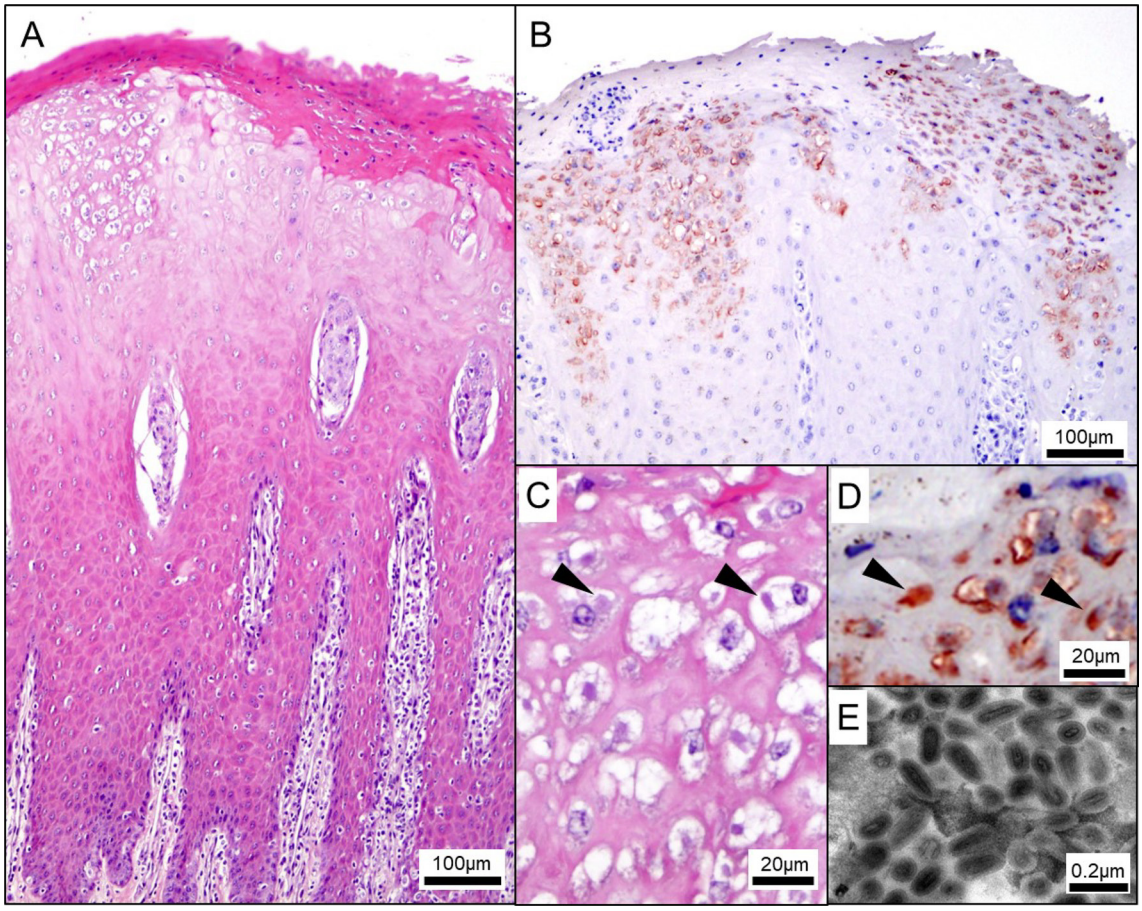

Fig. 2. Histopathology, immunohistochemistry and electron microscopic observation of the hard palate lesions from BPSV_SAGAbv6 obtained in 2020. **A**: Histopathology of the lesion of an affected calf. Vacuolar degeneration of spinous cells, and multifocal lymphocytes and macrophages mildly infiltrating the papilla is present. Hematoxylin and eosin (HE) staining. **B**: Immunohistochemistry of the same field shown in panel A. **C**: Intracytoplasmic inclusion bodies (arrowheads) are present in vacuolated spinous cells. HE staining. **D**: Immunohistochemistry of the same field shown in panel C. Parapoxvirus antigen is demonstrated corresponding to vacuolated spinous cells. **E**: Viral particles in the cytoplasm of an epithelial cell.

The phylogenetic classification using the NJ method was the same as phylogenetic classification performed using the ML method with a significant bootstrap value (data not shown).

On the hard palate from the BPSV_SAGAbv6, there were multiple erosions, a thickened keratinized layer and spinous cell layer around the erosions, and multifocal lymphocytes and macrophages mildly infiltrated the papilla (Fig. 2A). The spinous cells around the erosion showed balloon-like degeneration and necrosis, and amorphous eosinophilic intracytoplasmic inclusion bodies were found in the cytoplasm of these cells (Fig. 2C). Additionally, immunohistochemical staining showed anti-parapoxvirus rabbit serum-positive reactions consistent with the keratinized layer, spinous cells with balloon-like degeneration, and eosinophilic intracytoplasmic inclusion bodies (Fig. 2B and 2D). Transmission electron microscopy revealed a large number of elliptical virus particles with dense electron nuclei and envelopes in the cytoplasmic inclusions of spinous cells (Fig. 2E).

The oral papular stomatitis in this study exhibited histopathological findings consistent with a previous report, and was distinct from FMD, BVD-MD and IBR histopathology as it did not exhibit proliferative changes of spinous cells [26]. Furthermore, it was confirmed that this lesion was caused by parapoxvirus by using immunohistochemical staining and electron microscopy.

Stomatitis caused by parapoxviruses is not distinguishable by its etiological agents and it has recently been reported that BPSV can be detected or isolated together with PCPV [20, 25]. In this study, PCR and subsequent sequence analysis were performed on specimens obtained from 6 calves. Each nucleotide sequence was confirmed as the single nucleotide sequence of BPSV partial B2L gene by sequencing traces (data not shown). Therefore, the lesions were considered to be purely due to BPSV.

In this phylogenetic analysis, 554-nt of the partial B2L gene between PPP-1 / PPP-4 primers were analyzed. The phylogenetic tree of BPSV was divided into A- and B-lineages. Since the most strains from various regions belonged to the A-lineage, the A-lineage might be predominant lineage in the world and Japan. The B-lineage contained Japanese, French and USA strains. It is considered to be a viral strain that is spreading worldwide, though not as much as the A-lineage. Compared to the branch of A- and B-lineages, both the A- and B-lineages have more branches at the terminal of the phylogenetic tree. The concentration of many

branches having many locations within short gene distances is probably thought, due to worldwide transportations, that it is the result of the explosive spread of BPSV all over the world after a certain point in the past.

The distribution of BPSV in Japanese domestic cattle was shown to have a high positive rate (40–89%) nationwide in a large-scale serological survey in the 1990s using BPSV Chiba strain, probably cross-reacting with other parapoxviruses. In addition, the antibody prevalence in Saga prefecture in this survey is as high as 60% [24]. This study revealed that strains belonging to two respective lineages and one sub-lineage exist in Saga prefecture. In addition, no strains in this study belonged to the A2-sub-lineage, to which the known strain from Miyazaki belonged. In Saga prefecture, the proportion of fattening beef farmers is high, and the self-sufficiency rate of calves is chronically low (about 30%), so many farmers purchase calves for fattening from multiple areas of Japan, mainly from the Kyushu Island. The result that the BPSV field strains analyzed in this study belonged to several lineages may be related to the procurement practices for calves in Saga prefecture for a long time.

In this study, the genetic diversity of BPSV in Saga prefecture was clarified for the first time. Understanding the distribution and diversity of various field strains of BPSV may be useful for livestock hygiene improvement in predicting and controlling outbreaks of this disease. However, due to the lack of molecular epidemiological information on BPSV, further studies in various regions are required to control this disease.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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