NOTE *Virology*

Molecular Analysis of Parapoxvirus Detected in Eight Calves in Japan

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ABSTRACT. Molecular analysis of parapoxvirus envelope genes was performed. Parapoxvirus DNA was detected in eight calves from eight farms in Iwate Prefecture, Japan, between April and September 2010. Seven of the detected viruses were identified as bovine papular stomatitis virus (BPSV) by sequencing, because their nucleotide identity was more than 96.8% similar compared with BPSV strain V660. Among them, two formed a subgroup, because their amplicons were digested with *Xmn* I (a marker for BPSV) and *Hin*c II and exhibited a T61C nucleotide substitution in the sequenced region. The remaining virus was pseudocowpox virus that had not been reported previously in Japan. Our results demonstrate the presence of a new BPSV variant in Japan with genetic variability in the envelope gene.
KEY WORDS: bovine papular stomatitis virus, calf, molecular analysis, pseudocowpox virus.

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Parapoxvirus infections are widespread in ruminants, usually inducing mild clinical symptoms around the mouth or on the teats of affected animals [3]. These infections need to be differentiated from other serious diseases affecting the oral cavity and skin, such as foot-and-mouth disease, as clinical symptoms are similar [1, 6].

Known parapoxviruses (family *Poxviridae*) include orf virus (ORFV), bovine papular stomatitis virus (BPSV), pseudocowpox virus (PCPV) and parapoxvirus of red deer in New Zealand (PVNZ) [12]. Parapoxviruses were formerly classified based on natural host range, clinical symptoms and serology [13]; however, these criteria do not always reflect the molecular characteristics of these viruses [8, 17].

Amplification by polymerase chain reaction (PCR), sequencing and restriction fragment length polymorphism (RFLP) have been used for molecular characterization and classification of parapoxviruses [7, 8]. These methods have made it practical to study the prevalence of parapoxvirus infections in animals and to differentiate infections by other pathogens. Our aim was to determine unique molecular features of parapoxviruses detected in calves between April and September 2010 in Iwate Prefecture, Japan.

Affected calves (n=14) were 2- to 10-month-old and found on eight farms (farms 1–8). Calves presented with lesions (papule, erosion or ulcer) in the mucosa of the gingiva, lip, palate or tongue and in rare cases, excess salivation. No lesions were observed on teats or in the interdigital cleft of limbs. Clinical signs also included mild pyrexia (39.6–39.9°C) in three calves. Swab samples (n=10) were collected from mucosal lesions of eight affected calves and from the gingival mucosa of two calves without clinical symptoms that had been housed near calves with clinical signs. There were no obvious differences among the clinical features found in 14 affected calves. The eight farms were scattered throughout the prefecture, although two farms (farms 6 and 7) were located in the same town. There was no direct contact among the cattle and/or people between each farm.

PCR, RFLP, sequencing and phylogenetic analyses were performed on the swab samples [7, 8]. Viral DNA was extracted with a QIAampDNA Minikit (Qiagen, Tokyo, Japan), followed by PCR with primers (PPP-1 and PPP-4) [7] to amplify partial nucleotide sequences (594 bp) of the envelope gene. RFLPs were conducted with *Drd* I (marker for ORFV), *Xmn* I (marker for BPSV), *Pfl*M I (marker for PCPV) or *Hin*c II (marker for PVNZ). Following direct nucleotide sequencing, the deduced amino acid sequences were aligned, and phylogenetic analysis was performed using the Maximum Likelihood Method. Phylogenetic trees were constructed with the assistance of MEGA5 software. Nucleotide and amino acid sequences obtained were compared with each other with BPSV (V660; GenBank Accession No. AB044793), PCPV (VR634; AB044792) and PVNZ (DPV; AB044794) reference strains [7, 8] and with BPSV 9108 (JN162119) [2] and BPSV Iwate/bovine/2007 (AB538385) [15]. For detailed phylogenetic analyses, sequences of 12 previously reported virus isolates from Japan [8] and other countries [2] were used (AB044795–AB044801, JN171854, JN171860, JN171861, JN191576 and AY386265).

Virus isolation was performed from 10 swab samples as described above. Each sample was mixed with Eagle's minimal essential media (MEM) and then centrifuged for 30 min at 2,500 × *g*. The supernatant was inoculated onto bovine testis (BT) cells. The inoculated cultures were observed daily for at least seven days after inoculation. Cultures with no observable cytopathic effect (CPE) were passaged twice more in a blinded manner. After the virus was isolated, we performed PCR amplification of the envelope gene followed

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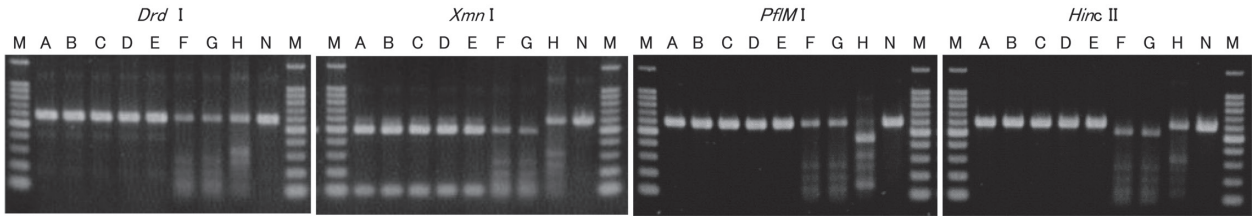

Fig. 1. RFLP analysis of eight amplicons. Restriction endonucleases used were *Drd* I, *Xmn* I, *Pfl*M I and *Hin*c II. Amplicons A–E and H were digested with *Xmn* I and *Pfl*M I, respectively. Amplicons F and G were digested with *Xmn* I and *Hin*c II. Lane M, 100 bp DNA ladder; A–H, digested PCR products; N, undigested amplicon (594 bp).

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BPSV      1   TGGTGTTGTTGGCGGCGTCGTCGCGGCCGGGGATGGAGAAGAGCCGCGTGGAGATGTCGATGCTCCCCACGCCGAAGTCGTTCAGCGTGCGCGCGGCCGA   100
Iwate/bovine/2007
          1   ..................................................................................................   100
A         1   ..................................................................................................   100
B         1   ..................................................................................................   100
C         1   ..................................................................................................   100
D         1   ..........A.......................................................................................   100
E         1   ..................................................................................................   100
F         1   ..................................................................C.........A.......................   100
G         1   ..................................................................C.........A.......................   100
9108      1   .T................................................................C.........A.......................   100
H         1   ............................C..T..CAC...CTTG...AC...C.....C.C...G.................G..AC............   100
PCPV      1   ............................C..T..CAC...CTTG...AC...C.....C.C...G.................G..GC............   100
PVNZ      1   ............C...........C..............C.......C...............G........A........G..............G.C   100
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BPSV    101   CACGGACAGCGGGTCCGCGTTCTTCCACTCCGTCACGATCACGCGGACACGCACGCTGCGGTCGATGGCCGCGCGCAGCAACGCGTCCATGATCCGCGGC   200
Iwate/bovine/2007
        101   ..................................................G.................A.................................   200
A       101   ...............T..................................G.................................................   200
B       101   ..................................................G.................A.................................   200
C       101   ...............T..................................G.................................................   200
D       101   ...............T..................................G.................................................   200
E       101   ..................................................G.................................................   200
F       101   .G.......................................C..G.......C.......................C.................   200
G       101   .G.......................PflM I.........C..G.......C.......................C.................   200
9108    101   .G.......................................C..G.......C.......................C.................   200
H       101   G.GC..G.................G..GGT...G.T...C..G.......C.....T.................G......G......T.....   200
PCPV    101   G.GC..G.................G..GGT...G.T...C..G.......C.....T.................G......G......T.....   200
PVNZ    101   ...C..G...........A........T..G..............C..G.......C...C....C..............G............GGCA...   200
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BPSV    201   CAGTACTCCACGCGGTCAGCGTGCCGGATCACGGGCACCAGGGACAGCAGCGACAGGTCGATGCTGTTCTTGGCGGAGTCGATGCGGTGCAGCACCAGGT   300
Iwate/bovine/2007
        201   ..................................................................C.............................   300
A       201   ..................................................................C.............................   300
B       201   ..................................................................C.............................   300
C       201   ..................................................................C.............................   300
D       201   ..................................................................C.............................   300
E       201   ...............................................G..................................................   300
F       201   ..................................................................T.......T........T.................   300
G       201   ..................................................................C.............................   300
9108    201   ..................................................................C.............................   300
H       201   ............GC.CTG......TT......C.......TC..G........G..............A..C...CTC.................G....   300
PCPV    201   ............GC.CTG......TT......C.......TC..G........G..............G..C...TTC.................G....   300
PVNZ    201   ..........T..T.CTG...........G........G.TC........A..G...........GAC...A....T.....................   300
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BPSV    301   CCTCGTCCAGCGTGCGGTAGAAGCCCAGGAACTTCTCGGGCGCGTCCGAGAAGAACACGCCGCCGCCCGCATGGTCCAGGTGGAAGTCCGTCGCCGTTGG   400
Iwate/bovine/2007
        301   ........................T.........................................................................G..   400
A       301   ........................T.........................................................................G..   400
B       301   ........................T.........................................................................G..   400
C       301   ........................T.........................................................................G..   400
D       301   ..................................................................................................G..   400
E       301   ..................................................................G.....................T.....G..   400
F       301   ..................................................G........G..............G....T.....................G..   400
G       301   ..................................................G........G..............G....T.....................G..   400
9108    301   ..................................................A........G..............G....T.....................G..   400
H       301   .......G.......................GCG...C....A............G...........G.AG....T...............G.....G..   400
PCPV    301   .......G.......................GCG...C....A............G...........G.AG....T...............G.....G..   400
PVNZ    301   ..........A...........T.................................G.T......T.....G....TG........A..G..G..G..G..   400
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Fig. 2.

Table 1. Percent nucleotide and deduced amino acid sequence identities for partial regions of the viral envelope

PCR products	A	B	C	D	E	F	G	H	BPSV	PCPV	PVNZ
Nucleotide sequence identities (%)											
A		99.6	100	99.6	98.9	96.6	97.1	85.7	98.9	84.8	86.1
B	100		99.6	99.3	98.9	96.6	97.1	85.7	98.9	84.8	86.1
C	100	100		99.6	98.9	96.6	97.1	85.7	98.9	84.8	86.1
D	100	100	100		98.9	96.6	97.1	85.7	98.9	84.8	86.1
E	100	100	100	100		96.8	97.1	85.9	98.9	85.0	86.8
F	96.2	96.2	96.2	96.2	96.2		99.5	85.4	96.8	84.5	86.1
G	96.7	96.7	96.7	96.7	96.7	99.5		85.9	97.1	85.0	86.5
H	83.7	83.7	83.7	83.7	83.7	83.7	84.2		85.4	98.6	83.6
BPSV	100	100	100	100	100	96.2	96.7	83.7		84.5	86.5
PCPV	82.6	82.6	82.6	82.6	82.6	82.6	83.2	98.9	82.6		83.2
PVNV	86.4	86.4	86.4	86.4	86.4	85.3	85.9	81.5	86.4	81.5	
Amino acid sequence identities (%)											

A to H, sequenced amplicons in this study; BPSV, bovine papular stomatitis virus strain V660; PCPV, pseudocowpox virus strain VR634; PVNZ, parapoxvirus of red deer in New Zealand strain DPV.

by RFLP as described above.

Parapoxvirus DNA was detected from eight swab samples (A–H) from six affected calves (farms 1–4, 6 and 8) and two calves without clinical symptoms (farms 5 and 7). Five amplicons (A–E) were cut with *Xmn* I only (496 and 98 bp fragments), and amplicon H was cut with *Pfl*M I (443 and 151 bp). Amplicons F and G were digested by both *Xmn* I (496 and 98 bp) and *Hin*c II (516 and 78 bp) (Fig. 1).

Nucleotide sequences of the PCR products and parapoxvirus reference strains are shown in Table 1. Two amplicons (E and G) were derived from two calves without clinical symptoms. Because the nucleotide sequences of these two amplicons were different from those of the other 6 amplicons that were derived from calves with clinical symptoms, we ruled out the possibility of contamination.

The location of the *Xmn* I site was conserved in BPSV V660, Iwate/bovine/2007, 7 of the amplicons identified in this study (A–F) and BPSV 9108 (Fig. 2). The *Hin*c II site in

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BPSV      401   CGTGATCATGGCGCAGCACAGCCGCGAGAAGGGGACCTTGGGCTCCACGATCATGGAGTAGAAGGTGTTGTACCGGTTCATCAGGTCCGTGGCCAGGTGC  500
Iwate/bovine/2007
          401   ..........................................................................................................  500
A         401   ..........................................................................................................  500
B         401   ..........................................................................................................  500
C         401   ..........................................................................................................  500
D         401   ..........................................................................................................  500
E         401   ..........................................................................................................  500
F         401   ..........................................................CG..........T.....................................  500
G         401   ..........................................................CG..........T.....................................  500
9108      401   ..........................................................CG..........T.............A.......................  500
H         401   .....CG.....................................................................G.......G.......C..........  500
PCPV      401   .....CG..........G.......T...C..C.....C.....................................G.......G.......C..........  500
PVNZ      401   .........C.A.........TG....T.........T.....................A.............G.......G.......C.......C..  500

BPSV      501   CGGTTGGTCGAGTACACGCCCAGGTTCTTGATGTTGGAGATGGAGCCGCCCGTG    554
Iwate/bovine/2007
          501   ................C.....................................    554
A         501   ................C.....................................    554
B         501   ................C.....................................    554
C         501   ................C.....................................    554
D         501   ................C.....................................    554
E         501   ................C.....................................    554
F         501   ..........................................................    554
G         501   ..........................................................    554
9108      501   ..........................................................    554
H         501   TT......G.....G.GC..G............G....C.C.............    554
PCPV      501   TT......G.....G.GC..G............G....C.C.............    554
PVNZ      501   TT......G.....G.G................G.TTG......C.....G...    554
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Fig. 2. (Left page and this page) Nucleotide sequence alignment of partial regions of the envelope gene. Nucleotides identical to BPSV are represented by dots. A–H, sequenced amplicons; BPSV, bovine papular stomatitis virus strain V660; Iwate/bovine/2007, BPSV strain Iwate/bovine/2007; 9108, BPSV strain 9108 strain; PCPV, pseudocowpox virus strain VR634; PVNZ, parapoxvirus of red deer in New Zealand strain DPV.

F and G was located upstream of the *Xmn* I site, because of the T61C nucleotide substitution. This substitution was also present in BPSV 9108. In the deduced amino acid sequences for F and G, the presence of an I301V substitution has the potential to confer a change in antigenicity. Generally, the virus envelope antigen is highly variable, because of its exposure to host immunological pressures. These substitutions might be the result of exposure to some as yet unidentified immunological pressures. The nucleotide sequence of amplicon B was identical to that of BPSV Iwate/bovine/2007 detected in a calf from Iwate Prefecture in 2007 [15]. Based on the phylogenetic tree we constructed, seven of the detected amplicons (A–G) could be considered BPSV; however, the sequence of amplicon H was most similar to that of PCPV (Fig. 3). Within the larger cluster, F and G formed a subgroup along with BPSV 9108. The nucleotide and deduced amino acid sequence identities for the amplicons and parapoxvirus reference sequences are shown in Table 1.

Three swabs from three calves (farms 1, 2 and 6) showed CPE in BT cell cultures after a second blind passage. Other swabs showed no signs of CPE. The molecular characteristics of three isolates were identical to those of amplicons (A, B and F) from the swab of the same calves.

Parapoxvirus envelope genes were detected in eight calves from eight farms. Six of these calves were symptomatic, and two were asymptomatic. Because the asymptomatic calves had been raised with one symptomatic calf at each farm, the calves might have been subclinically infected with the virus. Parapoxvirus infections with clinical symptoms in cattle have rarely been reported in this prefecture [15], although a high rate of antibodies positive against parapoxvirus has been found [14]. The increase in the level of detection is likely a consequence of increased surveillance by veterinarians and farmers following a foot-and-mouth disease outbreak in the spring of 2010 in Japan. This would suggest that parapoxvirus infections are widespread among cattle in Iwate. Infections occur irrespective of age with relapses common, because infections confer no significant immunity [5]. However, clinical symptoms were only observed in calves from 2- to 10-month-old. This finding is in accordance with a previous report that found that infections were more common in calves than adult cattle [5].

Five (A–E) of the eight samples examined in our study were classified as BPSV, based on amino acid identities with the corresponding regions in BPSV V660 [8]. In particular, amplicon B showed 100% nucleotide identity to the amplicon from Iwate/bovine/2007, in spite of being isolated in different years. This strongly suggests that this examined region encoding an envelope protein is highly conserved. By contrast, the presence of BPSV, which can be digested with *Xmn* I and *Hin*c II, was also indicated in this study. It was reported that BPSV 9108, isolated from a calf in France, contained a greater number of nucleotide substitutions in the envelope genes compared with other BPSV species [2]. Our phylogenetic analysis indicated that the French isolate could be grouped together with samples F and G (Fig. 3) and contained the same substitution at nucleotide 61 (Fig. 2). Our results also suggest that BPSV demonstrates a certain level

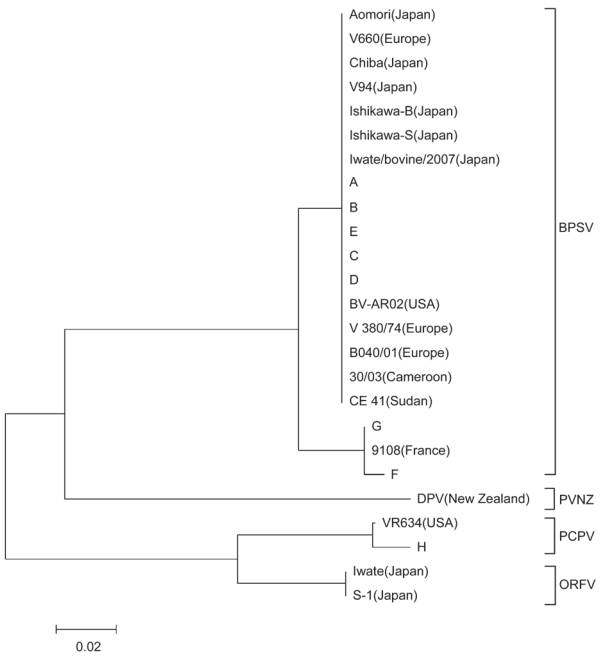

Fig. 3. Phylogenetic tree derived from the deduced amino acid sequences of partial envelope gene regions. A–H, sequenced amplicons; V660, BPSV strain V660; VR634, pseudocowpox virus strain VR634; DPV, parapoxvirus of red deer in New Zealand strain DPV; others, isolates from Japan and other countries.

of variability. Although there is no direct epidemiological evidence, these results suggest that BPSV variants are likely widespread among cattle in Japan.

Sample H was grouped with PCPV, based on RFLP results, nucleotide and amino acid identities and phylogenetic analysis. This is the first report describing PCPV in Japan. It was detected from the lesion around the mouth, not on the teats. The present result indicates that disease names are not always coincident with causal viral names in parapoxvirus infections as previously reported [8–10, 17]. PCPV VR634 was isolated from a human in the U.S.A. that had "milker's nodules" on the hands [4]. The high level of identity between sample H and PCPV VR634, whose origins are significantly different geographically, biologically and chronologically, is indicative of high levels of conservation in the envelope gene region we examined.

Recently, several case reports have highlighted the presence and wide distribution of parapoxvirus infections in cattle [9–11, 16]. Further molecular studies of parapoxviruses circulating among field animals are needed to clarify their epidemiology and pathogenesis.

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