Genetic heterogeneity among parapoxviruses isolated from sheep, cattle and Japanese serows (Capricornis crispus)

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Standard strains of four parapoxviruses and seven unclassified Japanese strains isolated from sheep, cattle and wild Japanese serows (Capricornis crispus) were compared molecularly. Restriction fragment length polymorphism (RFLP) analysis of viral DNA, indirect immunofluorescence assays using monoclonal antibodies, partial nucleotide sequencing of the envelope gene, phylogenetic analysis and PCR–RFLP were carried out. These analyses revealed that the parapoxviruses were divided into four groups and the region sequenced in this study was highly conserved within each group. Each of the Japanese isolates was classified into one of these groups. These findings also indicated that parapoxvirus infections among wild Japanese serows seem to be caused by at least two different parapoxviruses, bovine papular stomatitis virus and orf virus. The methods presented here are useful for genetic characterization and classification of parapoxviruses.

The genus Parapoxvirus in the family Poxviridae has four members, orf virus (ORFV), bovine papular stomatitis virus (BPSV), pseudocowpox virus (PCPV) and parapoxvirus of red deer in New Zealand (PVNZ). Parapoxvirus infections are widespread in ruminants worldwide. Parapoxviruses generally induce mild papular dermatitis around the mouth, teats and skin of affected animals such as sheep, goats, cattle and some wild species (Esposito et al., 1995; Fenner, 1996; Mayr & Büttner, 1990a, b, c; Mercer et al., 1997; Moss, 1996; Robinson & Lyttle, 1992). The lesions progress from papule/vesicle to pustule and scab formation over a period of about 1 month (Okada et al., 1986; Wheeler & Cawley, 1950). In some severe cases of stomatitis or dermatitis on teats, the lesions interfere with feeding or milking and these animals become unproductive. The viruses occasionally infect humans after close contact with skin lesions of infected animals or handling virus-contaminated materials. Milker’s nodule is known as one of the diseases in humans caused by parapoxvirus (Fenner, 1996; Mayr & Büttner, 1990b; Memar & Tyring, 1995; Robinson & Lyttle, 1992), and the infections are therefore classed as zoonoses.

In Japan, serological surveys have revealed that the morbidity of parapoxvirus infection is very high in sheep and cattle (Kuroda et al., 1999; Sentsui et al., 2000). Although several parapoxviruses have been isolated from sheep and cattle with or without clinical symptoms, these isolates have not been classified biologically and molecularly. Moreover, parapoxvirus infections in wild Japanese serows (Capricornis crispus) have been prevalent continuously in various areas since the 1970s (Inoshima et al., 1999, 2000b, 2001) and experimental transmission of parapoxvirus from the Japanese serow to cattle, sheep and goats has also succeeded (Ogino et al., 1996; Okada et al., 1986). The parapoxviruses circulating among Japanese serows have not yet been identified and the relationship between the virus in domestic animals and that in Japanese serows remains unclear. PVNZ was isolated from red deer in New Zealand and classified as a new parapoxvirus (Robinson & Mercer, 1995). Thus, it is possible that a new parapoxvirus may be distributed among wild Japanese serows.

The classification of parapoxviruses was formerly based on natural host range, clinical symptoms and serology (Robinson & Lyttle, 1992). However, some ORFVs were isolated from cattle and chamois rather than sheep (Robinson & Lyttle, 1992) and one strain was isolated from peripheral blood leukocytes of cattle rather than from teats or around the mouth (Sentsui et al., 1999). Moreover, they cross-react antigenically with each other and there are so far no established serological guidelines for their classification (Lard et al., 1991; Rosenbusch & Reed, 1983; Wittek et al., 1980). More recently, classification has been attempted according to restriction endonuclease analysis and DNA–DNA hybridization of viral DNA (Esposito et al., 1995; Fenner, 1996; Mercer et al., 1997; Moss, 1996; Robinson & Lyttle, 1992). Virus isolation is required for such studies; however, virus isolation is time-consuming and laborious and
In this study, ORFV strain NZ2 (Robinson et al., 1982), BPSV strain V660 (Menna et al., 1979), PCPV strain VR634 (Friedman-Kien et al., 1963) and PVNZ strain DPV (Horner et al., 1987; Robinson & Mercer, 1995) were used as standard strains of the four members of the genus *Parapoxvirus*. The four strains were kindly provided by A. A. Mercer (University of Otago, Dunedin, New Zealand). The Iwate strain was isolated from a sheep (Kumagai et al., 1971). Strains Aomori (Kumagai et al., 1976), Chiba (Kuroda et al., 1999), V94 (Sentsui et al., 1999) and Ishikawa-B (Kuroda et al., 1999) were isolated from cattle. Strains S-1 (Suzuki et al., 1993) and Ishikawa-S (Yata et al., 1996) were isolated from wild Japanese serows. Strains Ishikawa-B, Ishikawa-S and S-1 were kindly provided by T. Murakami (Nanbu Livestock Hygiene Service Station, Ishikawa, Japan) and N. Minamoto (Gifu University, Gifu, Japan).

Firstly, the strains were compared by restriction fragment length polymorphism (RFLP) analysis with the restriction endonuclease *KpnI* (Fig. 1), which is thought to be useful for molecular characterization of parapoxviruses (Mercer et al., 1997; Robinson & Lyttle, 1992). Viral DNA was extracted from infected FBM cells by alkaline lysis followed by phenol extraction (Inoshima et al., 2000a) or from the purified virus by sodium diatrizoate gradients (Inoshima et al., 1999). The four standard strains showed different DNA patterns. The Iwate strain and ORFV strain NZ2 showed the same restriction profile. All four strains isolated from cattle (Aomori, Chiba, V94 and Ishikawa-B) showed similar patterns to each other and the patterns resembled that of BPSV V660, even though some of these strains were isolated from teats or leukocytes rather than from around the mouth. Interestingly, the patterns of the two strains from Japanese serows were significantly different from each other. S-1 was similar to ORFV whereas Ishikawa-S was BPSV. The possibility of virus contamination was ruled out by RFLP of viral DNA with *SalI*, whereby different DNA patterns were shown among Japanese strains (data not shown).

Next, all 11 strains were tested for reactivity to six monoclonal antibodies (MAbs 21, 22, 23, 35, 37 and 40) against ORFV (Ueda, 1996) by an indirect immunofluorescence assay. These MAbs were kindly provided by N. Ueda (University of Otago, Dunedin, New Zealand) and K. Miyamoto (Wakayama Medical College, Wakayama, Japan). Strains NZ2, Iwate and S-1 reacted with all six MAbs; none of the other strains, including Ishikawa-S, reacted with any of the MAbs (Fig. 1). Similar to the RFLP analysis using *KpnI*, the two strains from Japanese serow showed different characteristics.

Next, partial nucleotide sequences (554 bp) of the envelope gene were determined by PCR amplification (Inoshima et al., 2000b) and direct sequencing. Sequences were obtained from both strands of each PCR product for verification. The sequences obtained in this study were submitted to DDBJ/EMBL/GenBank under accession numbers AB044792–AB044801. The sequence of ORFV strain NZ2 was obtained from accession number U06671. Neither deletions nor

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insertions in the nucleotide sequences were found between the strains. Overall nucleotide identities ranged from 82.3 to 100% (Table 1). High levels of identity were observed among isolates belonging to the same member of the genus, regardless of their geographical origin, host or year of isolation. The four Japanese isolates from cattle were 100% identical. The deduced amino acid sequences were aligned and compared (Fig. 2a; Table 1). Overall amino acid identity in the region ranged from 79-9 (between ORFVs and PVNZ) to 100% (ORFVs and BPSVs). Among the four standard strains, the identity ranged from 79-9 (between ORFV and PVNZ) to 95-1% (between ORFV and PCPV), indicating that there was a closer relationship in the region between ORFV and PCPV than among the other parapoxviruses. These results corresponded with previous results of Southern blot analysis (Inoshima et al., 2000b). Surprisingly, two strains (Iwate and S-1) showed 100% amino acid identity and six strains (Vööö, Ishikawa-S, Aomori, Chiba, V94 and Ishikawa-B) were completely homologous with each other, even though they were isolated independently from cattle, geographically, biologically and chronologically. This region is a homologue of the major envelope antigen of vaccinia virus and one of a limited number of ORFV proteins to which sheep mount a strong antibody response and which stimulate lymphocytes (Sullivan et al., 1994). The region also encodes an envelope protein in other viruses belonging to the family Poxviridae, such as molluscum contagiosum virus (Blake et al., 1991), fowlpox virus (Calvert et al., 1992), swinepox virus (Bárčena et al., 2000) and sheeppox virus (H. G. Heine, M. F. Rudd, A. J. Foord and D. B. Boyle, unpublished data). Generally, the virus envelope antigen is exposed to immunological pressure from the host and tends to be variable. Thus, our results suggest that this region is a highly conserved genetic marker of each parapoxvirus.

Phylogenetic analysis was performed by using the un-weighted pair group method using arithmetic averages (UPGMA) and a tree was constructed with GENETYX-MAC version 9.0 (Software Development Co.). On the basis of the derived tree, the parapoxviruses were divided into four phylogenetic clusters (Fig. 2b). The cluster of ORFV included Iwate and S-1. The cluster of BPSV included Ishikawa-S and the four strains from cattle.

The nucleotide sequence data allowed us to search for restriction enzyme recognition sites specific to a particular parapoxvirus. Drdl, XmnI, PflMI and HinclII were each able to differentiate one parapoxvirus from the others (Fig. 2c). High conservation of the envelope gene in each cluster may make it possible to differentiate parapoxviruses by PCR–RFLP. Taken together, these results indicated that Iwate and S-1 belong to ORFV whereas the other strains (Ishikawa-S, Aomori, Chiba, V94 and Ishikawa-S) belong to BPSV.

Since the lesions of the diseases caused by each of the four parapoxviruses exhibit almost the same phenotype and they cross-react antigenically, classification of parapoxviruses by natural host range and clinical symptoms is not definite and serological tests are not effective. This study represents the first phylogenetic analysis of the genus Parapoxvirus and demonstrates genetic heterogeneity within the genus. According to the criteria of parapoxvirus classification on the basis of host and the location of the lesion, Chiba and Ishikawa-B, isolated from cattle, would be classified into PCPV and V94, isolated from peripheral blood leukocytes, could not be classified. However, Chiba, Ishikawa-B and V94 were classified into BPSV. Ishikawa-S, which was isolated from the Japanese serow, was also classified into BPSV. Such contradictions were also observed with some isolates of ORFV isolated from cattle and chamois rather than sheep (Robinson & Lyttle, 1992). These findings suggest that classification of parapoxviruses by host and clinical symptoms does not always reflect the

<table>
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<tr>
<th>Strain</th>
<th>ORFV</th>
<th>BPSV</th>
<th>PCPV</th>
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<td>1</td>
<td>2</td>
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<td>1. NZ2</td>
<td>99.1</td>
<td>99.1</td>
<td>83.8</td>
<td>83.6</td>
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<tr>
<td>2. Iwate</td>
<td>98.4</td>
<td>100</td>
<td>84.1</td>
<td>83.9</td>
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<tr>
<td>3. S-1</td>
<td>98.4</td>
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<td>4. Ishikawa-S</td>
<td>83.2</td>
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<td>5. Vööö</td>
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<td>6. Aomori</td>
<td>83.2</td>
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<td>7. Chiba</td>
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<td>8. V94</td>
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<td>9. Ishikawa-B</td>
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<td>10. VR634</td>
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<td>93.5</td>
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Fig. 2. (a) Alignment of the deduced amino acid sequences of partial envelope regions of parapoxviruses. Amino acids identical to ORFV strain NZ2 at given positions are represented by dots. (b) Phylogenetic tree based on amino acid sequences of partial envelope regions of parapoxviruses. The tree was constructed by the UPGMA method by using GENETYX-MAC version 9.0. (c) PCR-RFLP analysis of parapoxviruses. A 594 bp fragment from the envelope region was amplified from each strain. PCR products were digested with DrdI, XmnI, PflMI and HincII. PCR products from ORFVs, BPSVs, PCPV and PVNZ were respectively cut only by DrdI (500 and 94 bp), XmnI (496 and 98 bp), PflMI (443 and 151 bp) and HincII (306 and 288 bp). Lanes: M, 100 bp ladder; 1, NZ2; 2, Iwate; 3, S-1; 4, Ishikawa-S; 5, V660; 6, Aomori; 7, Chiba; 8, V94; 9, Ishikawa-B; 10, VR634; 11, DPV.

classification obtained by molecular analysis. Therefore, the methods for PCR, sequencing and RFLP reported here will be useful for genetic characterization and classification of parapoxviruses, especially when the virus cannot be isolated.

We expected at first that the two strains from the wild Japanese serow might be classified into ORFV, because the Japanese serow belongs to the subfamily Caprinae and is more closely related taxonomically to sheep and goats than to cattle (Chikuni et al., 1995). However, although strain S-1 was classified into ORFV as expected, Ishikawa-S belonged molecularly and antigenically to BPSV. Likewise, Southern blot analysis of S-1 and Ishikawa-S indicated that they were heterologous (Inoshima et al., 2000 b). These findings suggest that at least two different groups of parapoxviruses, ORFV and BPSV, are circulating and cause disease among Japanese serows. Moreover, our data suggest that there are virus cycles between Japanese serows and domestic animals, such as from cattle or sheep to Japanese serows and vice versa. Further molecular analysis of parapoxviruses may reveal implications for pathogenesis and adaptation of the viruses in animals.

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References


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