

Isolation and Characterization of a Parapoxvirus from Sheep with Papular Stomatitis

Yukiko KANO^{1,2}, Yasuo INOSHIMA^{3,4*}, Tomoyuki SHIBAHARA¹,
Yoshiharu ISHIKAWA¹, Koichi KADOTA¹, Seiichi OHASHI³,
Kazuki MORIOKA³, Kazuo YOSHIDA³ and Shunji YAMADA³

¹ Hokkaido Research Station, National Institute of Animal Health
(Sapporo, Hokkaido 062–0045, Japan)

² Ishikari Livestock Hygiene Service Center (Sapporo, Hokkaido 062–0045, Japan)

³ Department of Exotic Diseases, National Institute of Animal Health
(Kodaira, Tokyo 187–0022, Japan)

Abstract

An outbreak of papular stomatitis occurred in a sheep herd in Hokkaido, Japan. Histological examination, immunohistochemistry, electron microscopic observation, and polymerase chain reaction (PCR) were carried out. Lesions were characterized by epithelial hyperplasia, acanthosis, ballooning degeneration of the spiny layer and stratum granulosum. A parapoxvirus was isolated from the skin lesion of affected sheep and characterized genetically and antigenically. Restriction endonuclease analysis of the PCR product showed an orf virus (ORFV)-specific pattern and the isolate reacted with monoclonal antibodies against ORFV. The partially deduced amino acid sequence of the viral envelope gene was identical to those of the major Japanese ORFVs. These results indicated that the outbreak was due to infection by parapoxvirus. The isolated virus could be classified into ORFV, and was closely related to the major Japanese ORFVs, but not foreign ORFVs or other parapoxviruses.

Discipline: Animal health

Additional key words: orf virus, outbreak

Introduction

Parapoxviruses cause papules, nodules, and scabs in the skin around the lips, nose, mouth, and teats of affected animals such as sheep, goats and cattle^{2,12,15}. In some severe cases, the papules/vesicles progress to pustules and ulcers. Parapoxviruses are often transmissible to humans and ‘milker’s nodule’ is known as one of the diseases in humans caused by parapoxvirus^{2,12,15}.

The genus *Parapoxvirus* in the family *Poxviridae* contains four members, orf virus (ORFV), bovine papular stomatitis virus (BPSV), pseudocowpox virus (PCPV) and parapoxvirus of red deer in New Zealand (PVNZ). The classification of parapoxvirus members is generally based on host range. Restriction fragment length polymorphism (RFLP) and cross-hybridization of viral DNA are also used¹⁵. We have previously demonstrated that

phylogenetic analysis of partial sequences of the viral envelope gene and polymerase chain reaction (PCR)-RFLP are useful for the differentiation of parapoxviruses⁶. Recent genetic studies suggest that seal poxvirus (SPV) should be included in the genus *Parapoxvirus*¹ but squirrel poxvirus may be classified into a different genus although the morphological features observed by electron microscopy are the same as for parapoxvirus¹⁸. Classification of parapoxviruses only by the natural host range is not definite in some cases. Some ORFVs were isolated from cases of bovine papular stomatitis in cattle and some BPSVs were isolated from sheep¹⁵. Moreover, both ORFV and BPSV cause pustular dermatitis in Japanese serows (*Capricornis crispus*)^{6,7} and both ORFV and PCPV also cause papular stomatitis in Finnish reindeer (*Rangifer tarandus tarandus*)¹⁹. In addition, experimental transmission of parapoxvirus from the Japanese serow to sheep, goats and cattle has

Present address:

⁴ Laboratory of Food and Environmental Hygiene, Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University (Gifu, Gifu 501–1193, Japan)

*Corresponding author: fax +81–58–293–2840; e-mail inoshima@cc.gifu-u.ac.jp

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succeeded^{11,13,14}. These results indicate that we can not differentiate parapoxviruses causing diseases in animals until genetic analyses are carried out.

An outbreak causing papules, pustules and scabs around the lips, nose and mouth occurred in a sheep herd in Hokkaido, Japan. In the present study, we report the parapoxvirus infection in the sheep herd and the histological and immunohistochemical features of the lesions. Moreover, an isolated virus was characterized by restriction endonuclease analysis, sequencing and monoclonal antibodies.

Materials and methods

1. Animals

On May 22, 2004, a sheep showing purulent scabs on the lips and nose was found in a herd in Hokkaido, the northern island of Japan. On May 25, 37 of 74 sheep (50%) in the herd were affected (Fig. 1). The herd was comprised of young sheep ranging from 1 to 2 years of age. They had papules, pustules, and purulent or dry scabs on their muzzles, lips, palpebrae, or nose. However, there was no bulla either in the tongue or oral mucosa in any sheep.

2. Examination for foot-and-mouth disease virus

It is important to distinguish parapoxvirus infection from foot-and-mouth disease (FMD), which is classified under list A of animal diseases by the Office International des Epizooties⁸, because FMD causes enormous economic damage to the animal industry and interrupts the international trade of animals and animal products. Therefore, at first, to deny FMD virus (FMDV), reverse transcriptase-PCR, antigen detection enzyme-linked immunosorbent assay (ELISA), and antibody detection ELISA for FMDV were carried out using mixed scabs from 7 sheep and each serum from 10 sheep as described previously¹⁶.

3. Histological examination

Several tissue samples taken from the lesions around lips of an affected sheep were collected, and fixed by immersion in 10% phosphate-buffered formalin and embedded in paraffin. The tissue sections (approximately 3 μ m thick) were stained with hematoxylin and eosin (HE) for the histological examinations.

4. Immunohistochemistry

For immunohistochemistry, a polyclonal antibody against parapoxvirus was prepared as follows. BPSV Chiba strain⁹ was purified from the supernatant of infected cells as described previously⁴. New Zealand

white rabbits were immunized hypodermically with the virus suspension in Gerbu Adjuvant LQ (Gerbu Biotechnik, Gaiberg, Germany) according to the manufacturer's instructions. To reduce non-specific reaction, rabbit sera were mixed with a powder of bovine spleen treated with acetone, centrifuged and confirmed for the specificity by an indirect immunofluorescence assay (IFA) using virus-infected or uninfected cells, and by immunohistochemistry using tissue sections from normal or affected animals prepared previously.

Serial tissue sections from an affected sheep were prepared for the streptavidin-biotin immunoperoxidase method (SAB) with Histofine SAB reagents (Nichirei Corp., Tokyo, Japan). After deparaffinization of the sections, endogenous peroxidase activity was blocked by methanol and 3% H₂O₂ (Sigma Chemical, St. Louis, MO, U.S.A.). The sections were lightly counterstained with Mayer's hematoxylin and assessed by light microscopy.

5. Electron microscopic observation

Small blocks taken from the 10% formalin-fixed tissue were postfixed 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-1010 transmission electron microscope (JEOL, Tokyo, Japan).

6. Virus isolation

Primary bovine testis (BT) cells were cultured in Eagle's minimum essential medium (Nissui, Tokyo, Japan) supplemented with 0.3% tryptose phosphate broth (Difco, MI, U.S.A.) and 5% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO₂ in air. After the examination for FMDV, suspensions containing 10% samples of the scabs in medium were inoculated into BT cells. Then the cells were passaged until cytopathic effects (CPE) were observed.

7. IFA

Cells showing CPE were tested for reactivities to monoclonal antibodies (MAbs) against ORFV. Six MAbs (21, 22, 23, 35, 37, and 40) against ORFV²⁰ were used in an IFA as described previously⁶.

8. PCR

DNAs were extracted from BT cells showing CPE or control cells using a DNeasy Tissue Kit (QIAGEN, Tokyo, Japan). PCR for parapoxvirus was carried out using a parapoxvirus-specific primer pair, PPP-1 and PPP-4 as described previously^{5,7}.



Fig. 1. Clinical representative of an affected sheep

9. RFLP

To identify the parapoxvirus species, the amplified PCR product was digested with restriction endonucleases, *DrdI*, *XmnI*, *PfI*MI and *HincII*.

10. Sequencing

Partial nucleotide sequences (554 bp) of the viral envelope gene were determined by direct sequencing using PCR products. Sequences were obtained from both strands for verification and compared to homologous sequences from other parapoxviruses, ORFV Iwate

(AB044795), ORFV Okinawa (AB080769), ORFV NZ2 (U06671), ORFV SA00 (AY278208), ORFV American vaccine strain (AY278209), ORFV European vaccine strain D1701 (AY453654), BPSV V660 (AB044793), PCPV VR634 (AB044792), PVNZ DPV (AB044794), and SPV SPV (AF414182).

Results

1. Examination for FMDV

Neither a specific PCR product nor a reaction to the FMDV antigen and antibody was detected from the suspensions of scabs and sera, respectively (data not shown).

2. Histological examination

Lesions were characterized by epithelial hyperplasia, acanthosis, ballooning degeneration of the spiny layer and stratum granulosum, and eosinophilic cytoplasmic inclusion bodies within vacuolated cells (Fig. 2A). In advanced parts of the lesions, numerous neutrophilic infiltrates into the superficial propria and epithelium were associated with erosion of the upper layers of necrotic cells. Focally extensive, severe, exuberant, perivascular to interstitial infiltration with histiocytes and lymphocytes was found in the submucosa. It was accompanied by fibroblastic proliferation and neovascularization.

3. Immunohistochemistry

Parapoxvirus antigen was detected using immunohistochemistry. The vacuolated epithelial cells were stained positively using a polyclonal antibody against parapoxvirus (Fig. 2B).

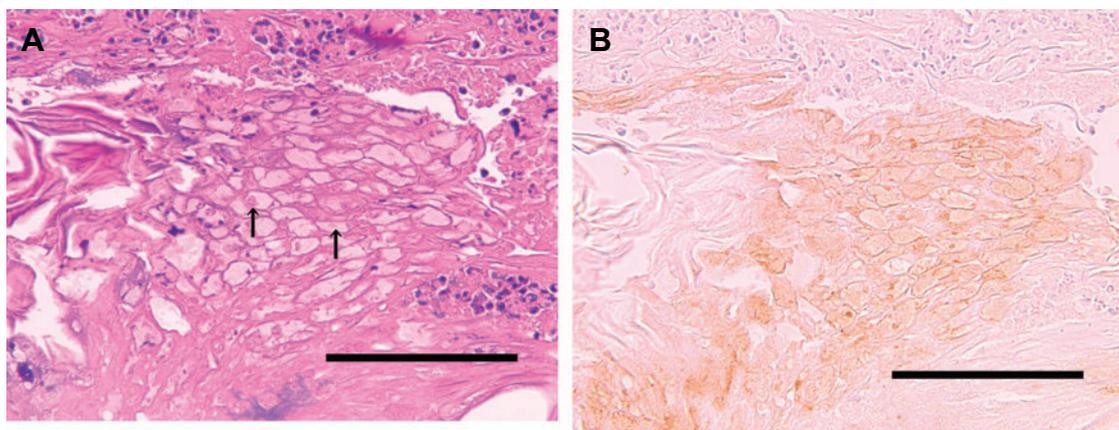


Fig. 2. Histology and immunohistochemistry of skin lesion of an affected sheep

- A: Histology of skin lesion of an affected sheep. Vacuolar degeneration of epithelial cells and necrotic debris are observed. Intracytoplasmic inclusion bodies (arrows) are seen in vacuolated epithelial cells. HE staining. Bar = 100 μ m.
 B: Immunohistochemistry of the same field shown in panel A. Parapoxvirus antigen is demonstrated corresponding to vacuolated epithelial cells. Bar = 100 μ m.



Fig. 3. Electron microscopic observation
Viral particles in the cytoplasm of an epithelial cell.
Bar = 200 nm.

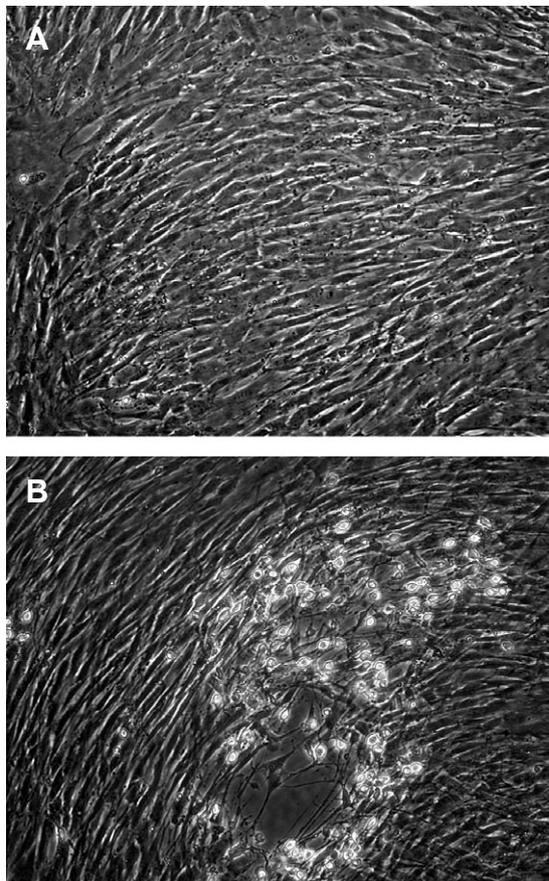


Fig. 4. Bovine testis (BT) cells showing cytopathic effects (CPE)
A: Normal BT cells. B: BT cells showing CPE.

4. Electron microscopic observation

Transmission electron microscopy disclosed the presence of many intracytoplasmic virions in the vacuolated epithelial cells (Fig. 3). Occasionally, virions were

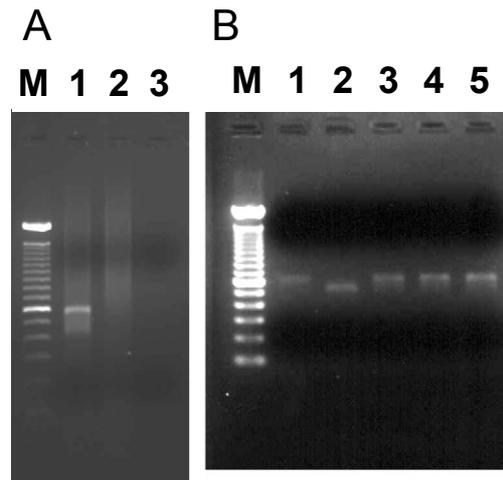


Fig. 5. PCR and PCR-RFLP

A: PCR for the detection of parapoxvirus DNA. Lane M: Marker. Lane 1: BT cells showing CPE. A specific 594 bp fragment was amplified. Lane 2: Control BT cells. Lane 3: PCR control using water as a template.
B: PCR-RFLP analysis for identification of parapoxvirus species. PCR product was digested with restriction endonucleases. Lane M: Marker. Lane 1: Buffer control digested without restriction enzyme. Lane 2: *DrdI* digestion. Lane 3: *XmnI* digestion. Lane 4: *PflMI* digestion. Lane 5: *HincII* digestion.

admixed with electron-dense material. The fine structure of the virions consisted of an oval- to dumbbell-shaped core surrounded by a membrane, lateral bodies, and a surface membrane. Particles had a length of approximately 250 nm and a width of nearly 150 nm.

5. Virus isolation and IFA

CPE was observed after blind passage (Fig. 4). We designated the isolate HIS (Hokkaido Ishikari Sheep) strain. All six MAbs reacted with BT cells showing CPE (data not shown).

6. PCR and PCR-RFLP

A specific 594 bp fragment was amplified from cells showing CPE (Fig. 5A). The amplified PCR product was digested only by *DrdI* (Fig. 5B). This RFLP pattern is a marker for the classification of ORFV⁶, indicating that the isolate HIS could be classified as ORFV.

7. Sequencing

The nucleotide sequence was determined from the PCR product. In comparison with other published sequence data, neither deletions nor insertions were found in the sequenced region. Compared to Japanese

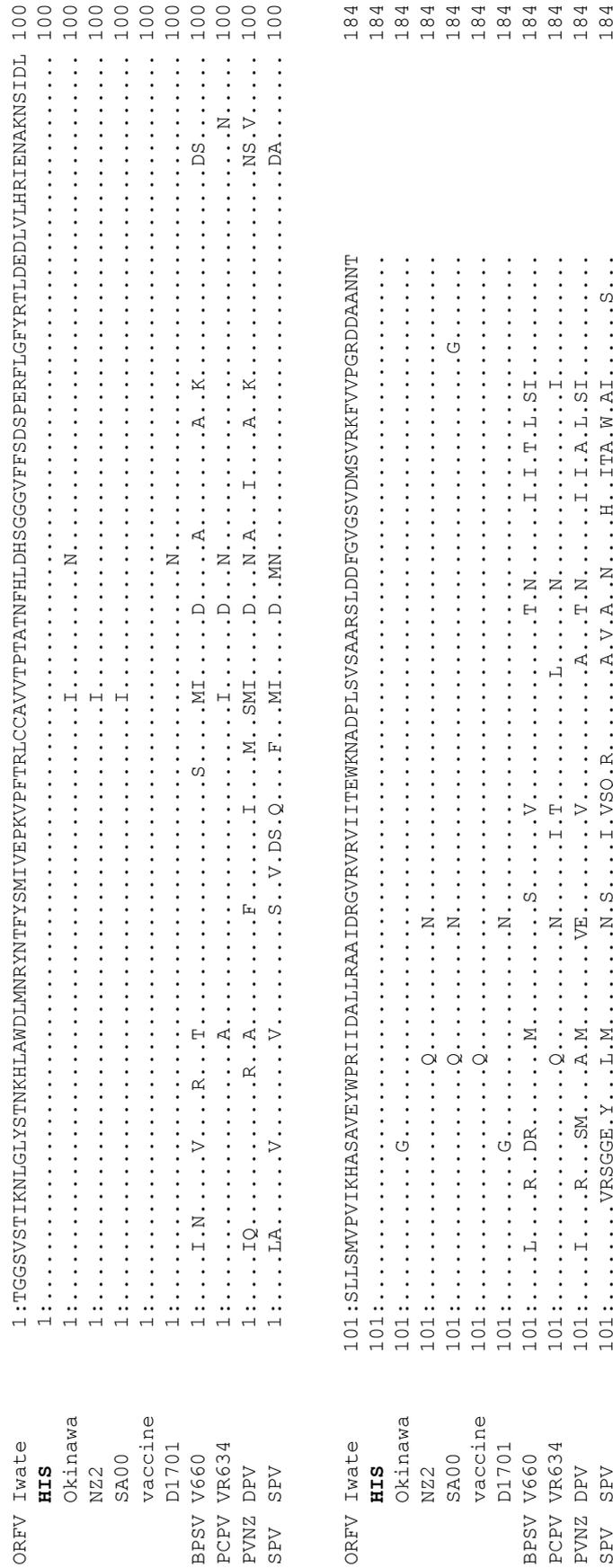


Fig. 6. Alignment of the partially deduced amino acid sequences of the viral envelope gene
 Amino acids identical to ORFV strain Iwate at given positions are represented by dots.

ORFV strain Iwate, only one substitution of a nucleotide was found but the deduced amino acid sequence was completely identical (Fig. 6). The sequence of HIS strain was submitted to DDBJ/EMBL/GenBank under accession number AB189670.

Discussion

Parapoxvirus infection exists all over the world and there is an old record about the infection in sheep in the late 17th century¹⁵. Generally, although the mortality of parapoxvirus infection is less than 1%, the morbidity in a single herd tends to be high as in this case. In Hokkaido, Japan, Kuroda et al. reported that the seroprevalence of parapoxvirus infection in sheep was 46/52 (88.5%)⁹. Parapoxviruses are closely related antigenically to each other; however, reinfection after recovery seems possible. Therefore, molecular characterization of the virus is important for the understanding and prevention of disease outbreaks.

Histological examination demonstrated proliferative skin lesions such as epithelial hyperplasia, acanthosis, ballooning degeneration of the spiny layer and stratum granulosum, and neovascularization, which are the histological hallmarks of the lesion of parapoxvirus infection. One of the reasons for the histological changes described above is that ORFVs have a viral vascular endothelial growth factor (VEGF) gene¹⁰. VEGF mediates endothelial cell proliferation, angiogenesis, and vascular permeability³ and ORFV VEGF is biologically active in sheep¹⁷. Indeed, inclusion bodies and the viral antigen were observed coincident with the histologically disordered area, suggesting that the virus might cause the skin lesions. With the results of histological and immunohistochemical examinations and observations by electron microscopy, as well as PCR and IFA with MABs, we diagnosed the disease as parapoxvirus infection.

PCR-RFLP analysis showed that the isolated virus, designated HIS, was classified into ORFV. We have previously sequenced a total of 15 ORFV isolates and viruses in tissue samples of sheep, goat and Japanese serows (*Capricornis crispus*) collected from 1970 to 2004 in various areas in Japan. The deduced amino acid sequences from most of them were completely the same^{6,7} and therefore we referred to the amino acid sequence of the ORFV Iwate strain as a consensus sequence for Japanese ORFVs. Interestingly, although the nucleotide sequence of the HIS strain was not the same as that of the Iwate strain, the deduced amino acid sequence of the HIS strain was identical to that of the Iwate strain, meaning that it was the same as the amino acid sequences of the major Japanese ORFVs.

Taken together, our results indicated that the outbreak was due to infection by parapoxvirus and the typical pathology of parapoxvirus infection was apparent in the lesions. Genetic and antigenic characterization indicated that the isolate HIS could be classified into ORFV and was very close to the major isolates of ORFV circulating in Japan, but not to foreign ORFVs or other species in the parapoxvirus genus.

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