Fowlpox Virus Infection in a Captive Japanese Rock Ptarmigan (*Lagopus mutus japonicus*)

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飼育ニホンライチョウ (*Lagopus mutus japonicus*) に認められた鶏痘ウイルス感染

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ABSTRACT. A female Japanese rock ptarmigan (*Lagopus mutus japonicus*) in captivity died of an unknown reason in 1985. The carcass was stored at -15°C until 2005 and then examined to define the cause of death. Finding a nodular lesion on the cere, avian poxvirus (APV) infection was suspected. An APV-specific 4b core gene sequence was detected in DNA samples prepared from lung, liver and the nodular lesion by PCR. The nucleotide sequence of the PCR product showed 100% identity to seven sequences of fowlpox virus (FWPV) previously reported. Integration of nearly intact reticuloendotheliosis virus gene which specifically found in virulent FWPV was also detected. These results indicate that the Japanese rock ptarmigan was died with FWPV infection.

Key words: avian pox, fowlpox virus, Japanese rock ptarmigan

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Rock ptarmigan (*Lagopus mutus*) has one of the widest distributions of any grouse species [1]. It is a sedentary species and ranges mainly across subarctic Eurasia and North America. One of the subspecies, Japanese rock ptarmigan (*Lagopus mutus japonicus*), inhabits in alpine zone at 1,800 – 3,000 m elevation in the central part of the main island of Japan. With an estimated population of only 3,000, Japanese rock ptarmigan has been designated as an endangered species (Appendix of the Law for Conservation of Endangered Species of Wild Fauna and Flora). It was also designated as a Special

Natural Monument under the Law for the Protection of Cultural Properties in Japan.

Omachi Municipal Alpine Museum in Nagano Prefecture raised Japanese rock ptarmigans from 1963 until 2005. We found a carcass of one Japanese rock ptarmigan stored at -15 °C in the museum in 2005. Museum keeper's records indicated that the bird was a female hatched artificially in July, 1980 and died in November 1985. Since the carcass was stored without pathological examination until 2005, we examined the bird to determine the cause of death.

Fig. 1 Japanese Rock Ptarmigan (*Lagopus mutus japonicus*) showing a nodular lesion on the cere.

In a postmortem examination, a nodular lesion was found on the cere near the upper beak (Fig. 1). Although the carcass was highly degenerated and could not be used for further pathological examinations including histopathological analysis, the nodular lesion implied an avian poxvirus (APV) infection.

APVs are members of the genus *Avipoxvirus* within the subfamily *Chordopoxvirinae* in the family *Poxviridae*. Currently, 10 defined species (canarypox virus, fowlpox virus (FWPV), juncopox virus, mynahpox virus, pigeonpox virus, psittacinepox virus, quailpox virus, sparrowpox virus, starlingpox virus and turkeypox virus) with three additional tentative species have been described in the *Avipoxvirus* genus [2]. Relationships among APVs were traditionally determined by examining the similarities in antigenic properties, host susceptibility, and cross-protection test among individual APVs. However, the nucleotide sequence of 4b core gene has recently been used to discern the relationships among APVs [3-5]. Consequently, we attempted to detect the APV-specific 4b core gene by polymerase chain reaction (PCR) as described below.

DNA samples were prepared from skin, lung and liver tissues with a SepaGene nucleic acid extraction kit (Sanko Junyaku, Tokyo, Japan) according to the manufacturer's instructions. The primers, P1 (CAG CAG GTG CTA AAC AAC AA) and P2 (CGG TAG CTT AAC GCC GAA TA) as previously described [6] were used. When the 4b core gene sequence was obtained by PCR, the nucleotide sequence of the PCR products were determined after cloning to pGEM-T Easy vector (Promega, Madison, WI, USA). At least three independent clones were sequenced by Dragon Genomics Center (TaKaRa Bio., Mie, Japan) with a Templiphi DNA Sequencing template Amplification Kit, DYEnamic ET dye terminator Kit, and MegaBACE1000 (all Amersham Biosciences, Tokyo, Japan). The sequences were analyzed with Genetyx-Mac version 13.1.1 (Genetyx, Tokyo, Japan) and assembled using Genetyx-Mac/ATSQ version 4.3.0 (Genetyx). The nucleotide sequence determined in this study was deposited in DNA data bank of Japan (DDBJ) as accession

number AB292467. Homology analysis of the nucleotide sequence data was examined by BLAST in DDBJ (<http://www.ddbj.nig.ac.jp/search/blast-e.html>).

PCR using DNAs prepared from nodular lesion, lung and liver obtained from the Japanese rock ptarmigan and the APV 4b core gene primers yielded a 578 bp fragment that was also observed in the positive control (Fig. 2). The nucleotide sequence of the PCR product trimmed the primer sequences (538 bp; DDBJ accession number AB292647) showed 100% identity with the sequences of seven APVs derived from chickens (accession numbers M25781, AJ005164, AY530302, AJ581527 and AY198100), pigeon (accession number AY530307) and turkey (accession number AY530304), which were previously classified genetically as FWPV [5]. These results indicate that the Japanese rock ptarmigan was infected with FWPV. The fact that the gene was also detected in the lung and liver suggests that the Japanese rock ptarmigan developed systemic infection.

Detection of the 4b core gene sequence showed that the Japanese rock ptarmigan was infected with FWPV. In 1975, the Alpine Museum used attenuated live vaccines derived from FWPV. This raised the possibilities that the detected virus was derived from one of the attenuated vaccines previously used. Consequently, a nearly intact provirus sequence of reticuloendotheliosis virus (REV) integration that is specifically found in pathogenic FWPV isolated in the USA [7] and Australia [8] was detected by PCR. We also found that the REV proviral sequence is integrated into FWPV in Japan (manuscript in preparation). The integration site primers [7], TR-1 (AAC AAT GAT ACG TCT CTT CC) and TR-2 (CAC ACG AAT ATA CCA ATA AGG) corresponding to regions in FWPV ORFs 201 and 203, respectively, and which flank the REV integration site of the FWPV genome were used (Fig. 3 (A)). Other primers for

Fig. 2 Detection of avian poxvirus-specific 4b core gene sequence by PCR from DNA samples prepared from lung, liver and a nodular lesion on the cere of the Japanese rock ptarmigan. A virulent FWPV previously isolated from chicken (unpublished) was used as a positive control. M, 100-bp DNA ladder; 1, positive control; 2, nodular lesion on cere; 3, liver; 4, lung; 5, negative control. Amplicon size is indicated on the right.

Fig. 3 Detection of a nearly intact REV provirus integration in FWPV genome by PCR. (A) Schematic representation of the relative positions of primers used in the PCR and the expected sizes of the PCR products. Each primer and the direction is indicated by a thick arrow. Each gene was mapped based on the nucleotide sequence reported by Singh *et al.* [7]. The nearly intact REV provirus sequence in the genome of FWPV is shown in gray. (B) Detection of REV provirus integration by PCR with primer pairs of TR-1 and Renv-R, and (C) Renv-F and TR-2. A virulent FWPV previously isolated from chicken (unpublished) was used as a positive control. M, 1 kb DNA ladder; 1, negative control; 2, DNA prepared from a nodular lesion on the cere; 3, positive control. Amplicon sizes are indicated on the right.

the REV *env* gene that lies within the proviral sequence, Renv-F (TCG ATT GCG GTA GCT CCA C) and Renv-R (CCA TCG AGA GTG ACA TTG C) designed by Singh *et al.* [9] were also used (Fig. 3 (A)). In this study, to determine whether the provirus sequence was integrated into the FWPV genome, we checked for it using primer pair TR-1 and Renv-R with an expected product size of 6.8 kb, and primer pair Renv-F and TR-2 with an expected size of 2 kb as shown in Fig. 3 (A).

Using the nodular lesion DNA as a template, PCR amplifications using the primer pair TR-1 and Renv-R, and the primer pair Renv-F and TR-2 yielded products of the expected sizes, approximately 6.8 kb (Fig. 3 (B)) and 2 kb (Fig. 3 (C)), respectively. This result indicates that a nearly intact REV provirus sequence corresponding to 8 kb is integrated in the genome of the FWPV infecting in the Japanese rock ptarmigan, and it must be a virulent field strain rather than the attenuated vaccine previously used.

APV infections were recorded in Japanese rock ptarmigan in the Omachi Alpine Museum in October, 1964 [10], September, 1969 [11] and November, 1986 [12]. Interestingly, all of the

outbreaks including the case in 1985 examined in this study occurred in autumn. Japanese rock ptarmigan molts in both spring and autumn. The captive Japanese rock ptarmigan in the museum usually molts between September and November, which corresponds with the disease outbreaks. The immune response of house sparrows (*Passer domesticus*) was found to be lowest when they completed molting, possibly as a result of the nutrient and energy deficits that accompany molting [13]. Thus, immunosuppression, as a result of molting, may be involved in the disease outbreak in Japanese rock ptarmigan.

Nucleotide sequence analysis of the 4b core gene and detection of the REV provirus sequence indicates that the captive Japanese rock ptarmigan was infected with FWPV. Although the natural host of the virus is known to be the chicken, no chicken farms were close to the museum and the source of the infection is unclear. However, It is known that FWPV is mechanically transmitted by blood feeding insects such as mosquitoes and biting midges [14, 15], which have been found around the museum. Because the captive rock ptarmigans were kept in wire cages which were open to outside

air, the disease might be transmitted from chickens to the museum by the blood feeding insects.

Our results suggest that Japanese rock ptarmigan is susceptible to FWPV infection. In central Africa, Latin America and Asia, insects and insect-borne diseases are spreading to higher elevation because of global warming [16]. In the alpine region of central Japan, which is the habitat of Japanese rock ptarmigan, the daily maximum temperature increased at an average rate of 0.6 °C /decade from 1965 to 2001 [17]. This would appear to increase the chance of wild Japanese rock ptarmigans being infected with FWPV by blood feeding insects in the future. To prevent the disease outbreaks and to save Japanese rock ptarmigan from extinction, blood feeding insects in alpine region of Japan may need to be continually monitored for the FWPV.

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要 約

市立大町山岳博物館で1985年に原因不明で死亡し、2005年まで冷凍保存されていた飼育ニホンライチョウ1羽の死亡原因を検索した。嘴の蟬膜に結節状病変を認め、禽痘ウイルス（APV）感染が疑われた。蟬膜病変部組織、肺および肝臓からPCRによりAPV特異的4b core遺伝子が検出された。増幅産物の塩基配列は、鶏痘ウイルス（FWPV）と100%一致した。FWPV病原株に特異的な細網内皮症ウイルスの配列も検出され、このライチョウがFWPV病原株の感染により死亡したことが推察された。

キーワード：禽痘、鶏痘ウイルス、ニホンライチョウ

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