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Molecular biological studies of rabbit hemorrhagic disease virus (Japanese isolate): *in situ* localization, molecular cloning and sequencing of the viral genome

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Rabbit hemorrhagic disease virus (RHDV), which belongs to the family *Caliciviridae*, causes a highly contagious and fatal disease in wild and domestic adult rabbits. The disease is manifested by acute necrotizing hepatitis and disseminated intravascular coagulation (DIC) in multiple organs.

In the present study, four New Zealand white rabbits aged six months were injected intramuscularly with an inoculum prepared from the frozen livers of rabbits that died of RHD in Shizuoka Prefecture in 1995. All the infected rabbits died within 26.5 to 46 hours after 2 to 3 hours of malaise, anorexia, and lethargy. Postmortem findings included an enlarged, pale, and friable liver with a fine lobular appearance, congestion and hemorrhage in the spleen and kidney, and several foci of petechial hemorrhages on the surface of the lung. Histopathological examination showed acute necrotizing hepatitis, congestion and DIC in multiple organs. In the liver, focal necrosis of the hepatocytes was predominantly found in the perilobular areas, while single-cell necrosis was scattered in the whole lobules. These clinical and pathological findings agreed with previous reports from other countries.

To investigate localization of the RHDV genes, *in situ* hybridization was performed using formalin-fixed, paraffin-embedded tissue sections of the liver, spleen, kidney, heart, lung, small intestine, submandibular and popliteal lymph nodes, and thymus of the experimentally infected rabbits. Digoxigenin (DIG)-labeled single-strand RNA probes were prepared by transcribing the cloned RHDV capsid protein (VP60) gene *in vitro*. Hepatocytes, Kupffer cells and macrophages in the spleen and lung showed intense signals in their cytoplasm, not in the nuclei. In the liver, the signal-positive hepatocytes were distributed mainly in the peripheral to intermediate areas of the lobules. In addition, the RHDV genes were frequently detected in the cytoplasm of the hepatocytes, which showed no cytopathic changes. These findings indicate that RHDV infects these cells *in vivo*. Furthermore, the intracytoplasmic localization of the RHDV genes suggested that viral replication and transcription exclusively occurred in the cytoplasm.

For molecular cloning of the RHDV genome, a cDNA library derived from the liver of the experimentally infected rabbit was screened by plaque hybridization using a DIG-labeled DNA probe that hybridizes to the polymerase region of the genome. A cDNA clone 3148 bases long was isolated and sequenced (Japanese RHDV isolate). The nucleotide sequence of the Japanese RHDV isolate, which corresponded to the region from nucleotides 4290 to 7437 of the German RHDV isolate genome, contained the VP60 gene and ORF2. Comparison of the nucleotide sequence of the Japanese RHDV isolate with the German, French and Spanish isolates revealed high degrees of similarity (94.2%, 93.8%, and 94.2%, respectively). The deduced amino acid sequences of the VP60 gene were also

closely related among the four isolates, although the Japanese RHDV isolate had six unique amino acid changes in the range from amino acids 299 to 480 in the VP60 region. I concluded that the RHDV isolates of four different countries share highly conserved nucleotide and amino acid sequences in the VP60 region, while there may be an internal hypervariable region as reported for feline calicivirus.

Annual and perinatal changes in fecal testosterone concentrations in Ezo Sika deer (*Cervis nippon yeoensis* HEUDE)

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The testosterone concentrations in both the feces and blood of Ezo Sika deer were examined during the annual and the perinatal periods. Moreover, the influence of preservation methods on the changes in fecal testosterone concentrations was also examined.

The subjects were stags (total n=4) and does (total n=13) kept at the Haiji farm in Hokkaido. The fecal and blood samples were collected from August, 1994 to August 1996. The fecal samples were frozen immediately after evacuation. The fecal and blood testosterone concentrations were assessed by radioimmunoassay.

When the values were transformed to common logarithms, correlation was shown between the testosterone concentrations in the feces and the blood collected in the stag (n=1) and does (n =5) during the annual season.

In the stag, the testosterone concentrations in both the feces and blood sharply increased late in August and peaked at the pre-breeding season (early October). They were decreased in November regardless of breeding behaviors that were maintained until February. In does, the testosterone concentrations in both the feces and blood were at lower levels than in the stag. But the fecal testosterone concentrations of the does showed small changes during the breeding season (from October to December). In parturient does (n=4), the fecal testosterone concentrations increased before parturition. In one non-parturient doe, no change was shown during the same period.

In the pregnant does (n=9), the fecal testosterone concentrations increased from about six weeks before parturition and decreased rapidly after parturition. They were higher than in the non-pregnant does (n=3) and the stags (n =3) during the same period.

The fecal testosterone concentrations increased when the feces were preserved for 48 hours at room temperature compared to those immediately preserved at −40°C after the evacuation. The testosterone concentrations were not increased when the feces were preserved with ethanol or antibiotics for 48 hours at room temperature.

In conclusion, the fecal testosterone concentrations were correlated (male r=0.93, female r =0.71) to those of blood in Ezo Sika deer. Moreover they showed similar patterns during the annual cycle. The testosterone concentrations increased during the pre-partum period. It