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Research article

# Risk of hepatitis E virus infection associated with urban invasion of wild Deer (*Cervus nippon yesoensis*) in Hokkaido, Japan

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## Abstract

The number of hepatitis E cases in Japan is reported to be high in Hokkaido, where small outbreaks, mainly of hepatitis E virus (HEV) genotype 3, have been reported over the past 15 years. In Hokkaido, the wild deer population is increasing and is found in mountainous areas and urban areas, where civilians mix with wild deer. Since cases of HEV transmission from deer to humans have been reported, this study aimed to determine the prevalence of the hepatitis E virus in Hokkaido, Japan, due to the increasing urbanization of the deer habitat. The study examined the detection of HEV RNA in the liver, and the distribution of HEV RNA prevalence among 153 wild deer was significantly higher in deer captured in urban areas (42.7%) than in mountainous areas (17.2%). HEV-ORF2 sequencing of positive deer revealed that all sequences belonged to the same cluster in genotype 3. The same strain of HEV was circulating among the deer. Relative risk (RR) was analyzed for the risk of HEV infection in different deer capture areas in urban and mountain areas, with an RR value of 2.479 and a 95% confidence interval of 1.089 - 5.646, and a chi-square test (×2 test) result of 0.01. The results suggest that these deer may be a risk factor for zoonotic disease in human habitats.

**Keywords:** Hepatitis E virus, Hokkaido sika deer, Epidemiology, Zoonosis, Public health, Transmission risk

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## Introduction

Hepatitis E virus (HEV) is a causative agent of acute hepatitis in humans. The transmission mode is orlay by ingesting food or water contaminated with HEV. HEV is a linear, plus-stranded, single-stranded RNA virus with no envelope and an icosahedral structure of 27-34 nm. The genome size is approximately 7.2 kb and has three open reading frames (hereafter ORFs), arranged in the order of ORF1, ORF3, and ORF2 from the N-terminal side. ORF1 is a non-structural protein consisting of approximately 1693 amino acids, and ORF2 is a capsid protein comprising 660 amino acids. HEV is classified in the family *Hepeviridae*, subfamily *Orthohepevirinae*, and genus *Paslahepevirus*. *Paslahepevirus balayani* is divided into eight genotypes (Kenney, 2019; Purdy et al., 2022). Genotypes 1 to 4 are the main types that infect humans, and types 3 and 4 are the zoonotic agents that pose public health and medical problems. HEV genotype 3, which has been reported in humans and deer, has been further classified into subtypes, and genotypes 1 to 4 are the major types of infection in humans (Aslan et al., 2020; Lhomme et al., 2016; Wang et al., 2018). Quasi-enveloped HEV (eHEV) particles are covered with a host-derived lipid membrane. Such particles are found in the serum of HEV-infected animals after virus release via the exosome pathway on the basolateral side of hepatocytes and in cell culture supernatants of infected cells (Nagashima et al., 2017; Takahashi et al., 2010; Yamada et al., 2009). When the virus is released apically into the bile duct, the lipid membrane is removed by the detergent effect of bile acids, and non-enveloped HEV (nHEV)

particles are then released into the environment through the intestine (Yin et al., 2016).

Hokkaido is the northernmost of Japan's four main islands, with an area of 83,450 km². Surrounded by the Pacific Ocean, the Sea of Japan, and the Sea of Okhotsk. Hokkaido comprises mountains, vast wetlands, and natural lakes. The climate is cool, low, and humid. The island is inhabited by various wildlife, including bears and deer, and human activities include agriculture, forestry, and fishing. The Japanese deer is a member of the even-toed ungulate family *Cervidae* and is the largest of the subspecies *Cervus nipponensis* in Hokkaido. The deer is an herbivore that inhabits forests, grasslands, and forest margins, feeding on weeds, leaves, and twigs from broadleaf forests, grasses, and agricultural crops. In winter, they dig up and eat grass, bamboo grass under snow, bark, and branches. They are highly fecund, and in recent years, due in part to mild winters, the natural mortality rate has been declining, and their population has been increasing significantly. The increase in population has been accompanied by an expansion of the species' active range, which has risen to an estimated maximum of 720,000 deer (Hokkaido Government, 2023; Kaji et al., 2022).

As the deer population has increased in recent years, there has been a noticeable increase in deer wandering into residential areas and mountainous regions, and there are more opportunities for deer to come into contact with people, damage agricultural crops, and cause traffic accidents. The Hokkaido government office has recently enacted the Hokkaido Sika Deer Control Promotion Ordinance as a deer habitat adjustment measure. At the same time, the Hokkaido government also encouraged the consumption of venison (Natural Environment Bureau, 2024). The presence of hepatitis E in deer has been confirmed in Hokkaido, and the number of hepatitis E patients in Hokkaido has been reported to be significantly higher than in other areas of Japan (National Institute of Infectious Diseases, 2024). Small outbreaks of hepatitis E cases, mainly HEV genotype 3, have been reported in the past 15 years (Sakata et al., 2021). On the other hand, information on deer habitat and HEV prevalence remains unclear. The aim was to determine the distribution of HEV among deer habitats and associated risk factors.

## Material and methods

### Hokkaido Sika deer liver samples

The livers of 153 Sika deer were collected from animals captured in northeastern Hokkaido, Japan, five years before COVID-19 onset (2014-2019). The number of Hokkaido Sika deer hunted in the study area during the five years was approximately 14,000, and the sample size was 146 deer when the confidence level in the survey was set at 95%. Therefore, a comparative analysis was conducted with 153 deer in the study. The distribution of deer habitat was divided into mountain deer (n=29) and deer in urban areas (n=124) according to the hunting areas. Deer hunting areas were identified on a 5 square km hunting map and divided into mountainous and adjacent to residential areas. Hunting was conducted by local hunters in accordance with the Hunting Regulation Law of Japan (Article 2, Paragraph 8 of the Wildlife Protection and Hunting Management Law, Ministry of Environment, Japan) (Hokkaido Government Map of Areas, 2023). Table1 shows a breakdown of the samples. One hundred mg of liver collected within four hours after hunting was aseptically collected in tubes and crushed by Tissue Lyser II (QIAGEN, Hilden, Germany) with zirconia beads and 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA). The homogenized liver samples were stored at -30°C until examination.

**Table 1:** Composition of heads by age group and sex of deer under study.

| Age Group* | Male | Female | Total | % |
|---|---|---|---|---|
| <1 year old | 13 | 6 | 19 | 12.4 |
| 2-3 years old | 23 | 71 | 94 | 61.4 |
| ≥ 4 years old | 26 | 14 | 40 | 26.1 |
| Total | 62 | 91 | 153 | |

*The age was assessed by observing the degree of branching of the horns and the annual rings (layers) of the cementum part of the gingival root.

**RNA extraction from the liver and detection of HEV RNA and the sequence analysis**

Liver samples completely dissolved in TRIzol reagent using the Tissuelyzer were allowed to stand at room temperature for 5 min to ensure complete separation of the nucleoprotein complex. 0.2 mL of chloroform was then added per mL of TRI reagent, shaken vigorously for 15 seconds, allowed to stand at room temperature for 10 min, and centrifuged at 12,000 ×g for 15 min at 4°C. The aqueous phase (0.5 mL) of the centrifuged supernatant was transferred to a new tube, mixed with 0.5 mL of 2-propanol, allowed to stand for 10 min, and centrifuged at 12,000 xg for 10 min at 4°C. RNA precipitation was confirmed, the supernatant was discarded, 1 mL of 75% ethanol was added and vortexed, centrifuged at 12,000 ×g for 5 min at 4°C, the supernatant was discarded, and the RNA pellet was air dried for 10 min, dissolved in DNase/RNase-free $H_2O$ and used for HEV-RNA ORF2 detection. Primer sequences and PCR conditions used for RT-PCR and nested-PCR are shown in Table 2. One Step RT-PCR Kit (QIAGEN, Hilden, Germany) was used, while Nested-PCR was performed using TaKaRa ExTaq (Takara Bio Inc. Shiga, Japan). The nested-PCR products were electrophoresed using a 1.5% agarose gel to detect the amplified bands and were purified by FastGene Gel/PCR Extraction Kit (NIPPON Genetics Co., Ltd., Tokyo, Japan). Then, they were sequenced by ABI Prism Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosciences, Foster City, CA, USA). The sequences were analyzed using 3500 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA) and compared with other sequences of ORF2 genes from the NCBI GenBank database. MEGA software (version X, https://www.megasoftware.net) was used for multiple alignments and phylogeny tree construction. The phylogenetic tree was constructed using the neighbor-joining method based on the partial nucleotide sequences of other samples using previously reported sequences genotypes 1-4. The evolutionary history was inferred using the Maximum Likelihood method and the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood is shown. The initial tree for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of estimated pairwise distances using the Tamura-Nei model. Then, the topology with a superior log likelihood value was selected. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 24 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There was a total of 290 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). A list of HEV genotypes with sequence comparison of HEV-ORF2 regions is shown in Table 3.

**Geographic information**

Based on a map by the Geospatial Information Authority of Japan, the study area in Hokkaido is shown in the frame. The central part of the region is mountainous, and the seashore is inhabited, as indicated by color-coded contour lines (Figure 1). Deer hunting areas were identified on topographic maps based on a 5 square km grid division (mesh data) on the captured map to determine the location of deer on the map. The Mesh data is a regional mesh defined as a longitude/latitude grid on a map. To guide the location of the specified hunting equipment prohibition area, this map illustrates a 5 km mesh of the deer huntable area based on map information from the Geographical Survey Institute (Approval No. R4JHs 53-GISMAP55604). Based on the topographic map, residential and agricultural areas were shown in light green, while red was classified as rural-urban and white as wooded mountainous (Hokkaido Government, 2023).Deer habitat areas were defined as mountainous and urban areas adjacent to mountains (Figure 1).

**Table 2:** Primer sequences used for RT- nested PCR

| Target | Step | Primer | Sequence | Annealing temperature |
|---|---|---|---|---|
| ORF2 | RT-PCR | F1 | 5'-AATTATGCYCAGTAYCGRGTTG-3' | 55℃ |
| | | R1 | 5'-CCCTTRTCYTGCTGMGCATTCTC-3' | |
| | Nested-PCR | F2 | 5'-GTWATGCTYTGCATWCATGGCT-3' | 55℃ |
| | | R2 | 5'-AGCCGACGAAATCAATTCTGTC-3' | |

R = A or G, Y = T or C, K= T or G, W = A or T, M = A or C; (Huang et al., 2002)

**Table 3**: List of HEV genotypes with sequence comparison of HEV-ORF2 regions.

| Genotype | Animals | Accession number |
|---|---|---|
| 1 | Human | AB513614、KX398678、AJ428851 |
| 2 | Human | M74506 |
| 3 (in Japan) | Human | AB107368、AB115541、AB082566、LC202065 |
| | Pig | AB094272、AB194488、LC458774、AB194523 |
| | Boar | AB180053、AB189070 |
| | Deer | AB189071 |
| 3 (in Hokkaido) | Human | AB807428、AB105892、AB671050、AB434136、AB670973 |
| | Pig | AB105903、AB679635 |
| | Water | AB679637 |
| 3 (in other country) | Human | KJ742846、LT745945、HM446630、MK140443、JX912476、KT833800 |
| | Pig | MH003296、AB714129、FJ600536、KP966827、KC145147 |
| | Boar | MG739305、FJ718694 |
| | Deer | MG739311 |
| 4 | Human, Macaca | AB671146、GQ242160、EU332150 |

## Results

### Detection of HEV-RNA in liver

In this study, the deer samples consisted of 41.7% males and 35.5% females. In terms of age composition, 35.3% of the deer were between 0-1 years old, 38.9% were 2-3 years old, and 36.6% were 4 years old or older. No significant differences were observed based on age. The prevalence of HEV in the deer livers showed that 58 out of 153 deer (37.9%) tested positive. When comparing results by capture location, 5 out of 29 deer (17.2%) in wooded mountainous areas were found to be positive, while 53 out of 124 deer (42.7%) in urban areas tested positive. This indicates a significantly higher prevalence of HEV in urban deer compared to those in wooded mountainous areas (Figure 2). Relative risk (RR) was analyzed for the risk of HEV infection in different deer capture areas in urban and mountain areas, with an RR value of 2.479, a 95% confidence interval of 1.089 - 5.646, and a chi-square test (×2 test) result of 0.01. Therefore, there is a concern that the entry of deer into urban areas increases the risk of HEV infection and the associated frequency of human interactions. The study area is indicated by a black frame, and the color classification of the areas of human habitation and production activities (e.g., agriculture) and mountainous areas are shown in the upper right frame. Light green indicates residential and agricultural areas, white indicates wooded mountainous areas, and red indicates regional cities. Topographic information was collected from maps provided by the Geospatial Information Authority of Japan (GSI). The vertical axis shows the percentage of positive deer, and the horizontal axis shows the capture area. Values in parentheses indicate the proportion of HEV-positive deer in the deer activity area.

### The sequence analysis of the HEV-RNA ORF2 region

From the positive samples for HEV-ORF2, six deer were examined for ORF2 sequencing analysis; the breakdown of the six animals was urban (#23, #165, #179) and mountain (#234, #382, #398), respectively. The partial ORF2 nucleotide identity among the six samples analyzed showed 94-100%. The phylogenetic tree was constructed using the neighbor-joining (NJ) method based on the partial nucleotide sequences of other samples using previously reported sequences (Figure 3). The HEV sequences used for phylogenetic analysis are listed in Table 3. Based on the results of phylogenetic tree analysis, the HEVs isolated in this research #23L2 (LC836370), #165L2 (LC836371), #179L2 (LC836372), #234L2 (LC836373), #382L2 (LC836374), and #398L2 (LC836369) were in the same cluster and belonged to genotype 3.

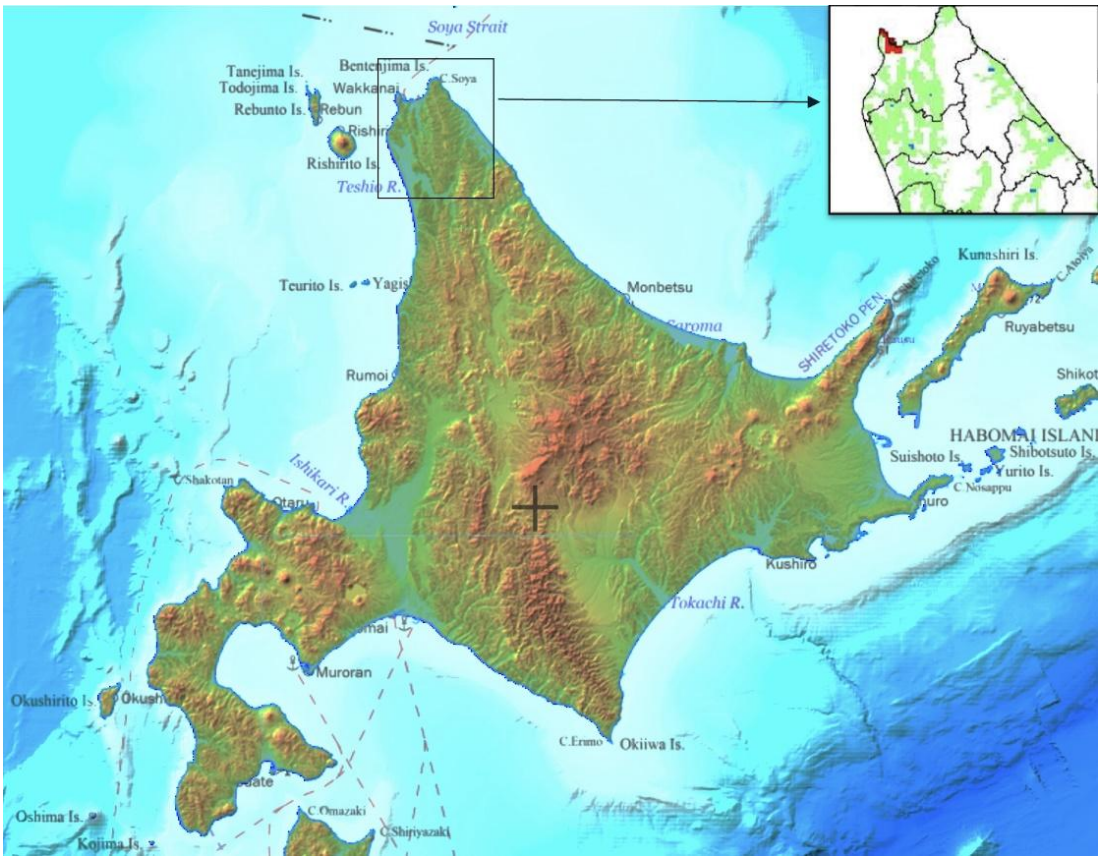


**Figure 1:** The deer hunting area is located in the northern part of Hokkaido, Japan.

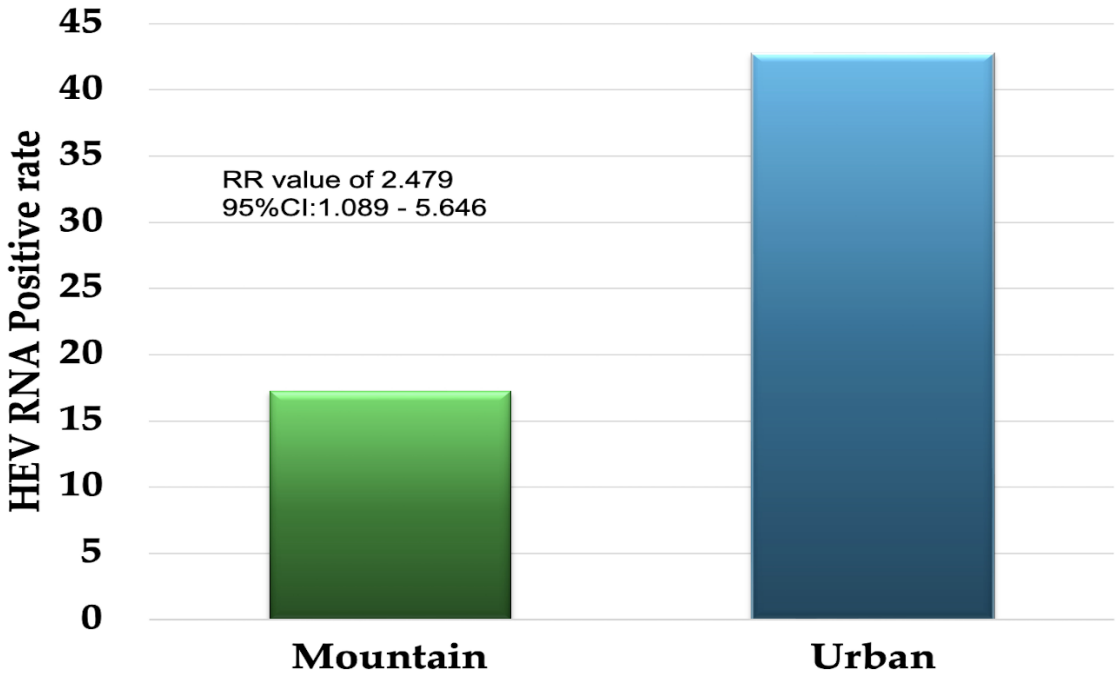


**Figure 2:** Prevalence of HEV-RNA-positive deer by location.

The results from the Maximum Likelihood method showed deer HEV is circulated within deer. Marks after the name of the sequenced sample indicate: red arrowhead, six isolated HEV from deer; blue arrowhead, isolated from humans in Hokkaido; orange arrowhead, isolated from pigs in Hokkaido; and green arrowhead, isolated from deer on land other than Hokkaido. Genotypes (1, 3) and subtypes (G3a, G3b) are shown on the right. Based on the phylogenetic tree analysis, all deer HEV sequences belonged to genotype 3. The bar shows 0.02 nucleotide substitutions per site.

The evolutionary history was inferred using the Maximum Likelihood method and the Tamura-Nei model (Figure 4). The bootstrap consensus tree inferred from 1000 replicates represents the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. This analysis involved 41 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. There was a total of 290 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. Arrow: six isolated HEV from deer in this research.

**Discussion**

The research clearly shows the differences in HEV positivity rates among different deer habitats. HEV-RNA prevalence was 37.9% (58 of 153) in total deer samples. Previous reports on the seroprevalence of Sika deer in Hokkaido in 2007 indicated an antibody-positive rate of 1.2%. Additionally, a 2012 study on HEV infection rates found no positive cases (Ishida et al., 2012; Matsuura et al., 2007). Although differences in the number of deer and study protocols preclude simple comparisons with previous reports, our findings suggest that HEV is widespread and infection is maintained in deer herds. This research also notes the high HEV-positive rate of deer in urban areas bordering mountainous areas.

The relative distribution density of the deer increases with the population, which is higher in winter as they aggregate and live in large herds, increasing the opportunity for virus circulation between deer (Igota et al., 2004). It is also possible that the detection efficiency may have increased due to the rapid collection and storage of test samples at deer slaughter facilities during the collection, which ensured good quality samples.

When compared by capture area, HEV prevalence was significantly higher in urban areas than in mountainous areas. The above epidemiological findings suggest that probabilities for HEV infection increased among deer that lived in herds during the winter, and infected deer appeared in urban areas in search of food when it was scarce in the mountains due to snowfalls. Recently, there has been concern about the increasing direct contact with Hokkaido Sika deer in the human habitat. Damage to agriculture and forestry and the high number of traffic and train accidents have increased due to their presence in urban areas (Rural Development Bureau, 2024).

In the evolutionary analysis of deer, the HEV by maximum likelihood method was classified as genotype 3. No difference in the HEV prevalence rate between sexes or ages was observed in the study. In addition, no significant difference was observed between age groups, confirming that the virus infects animals from 1 year of age and older than 4 years. In animals having normal immune systems, HEV infection is transient without clinical symptoms. It does not persist in infection with HEV, suggesting the possibility that recurrent infection may occur within the deer herd. It may be related to their lifestyle in groups during the winter. This inference requires further detailed investigation. It is noted that these viruses may be transmitted among deer.


**Figure 3:** NJ Phylogenetic tree of hepatitis E virus (HEV) ORF 2 region. Red arrowhead indicate HEV from deer; blue arrowhead indicate isolates from humans in Hokkaido; orange arrowhead indicate isolates from pigs in Hokkaido; and green arrowhead indicate isolates from deer on land other than Hokkaido.

Strains isolated from deer in Honshu Island were responsible for hepatitis E caused by undercooked wild deer meat in Hyogo Prefecture in 2003 (Tei et al., 2004; Takahashi et al., 2004). The results suggest that a two-stage interspecies HEV transmission may have occurred, with transmission among wild animals and then to humans [23]. However, as wild boars do not inhabit Hokkaido, HEV transmission in the wild is thought to be maintained between deer.

This research showed that the relative risk (RR) analysis of the higher HEV prevalence in deer entering urban areas than in mountain forests showed a value of 2.7 or higher, indicating an increased risk of HEV infection in deer entering urban areas. The HEV detected in the study area was a zoonotic genotype 3 virus. These results suggest that urban areas with high population densities are more susceptible to contamination from the feces and movement of HEV-infected deer. There is increased exposure to infected deer feces and the potential for direct and indirect transmission; HEV is relatively stable in the environment, and there may be a continuing risk of transmission through water and food. A related concern is that the feces of HEV-infected deer can contaminate water sources and agricultural lands, increasing the potential for ingestion of this HEV-contaminated water and crops.

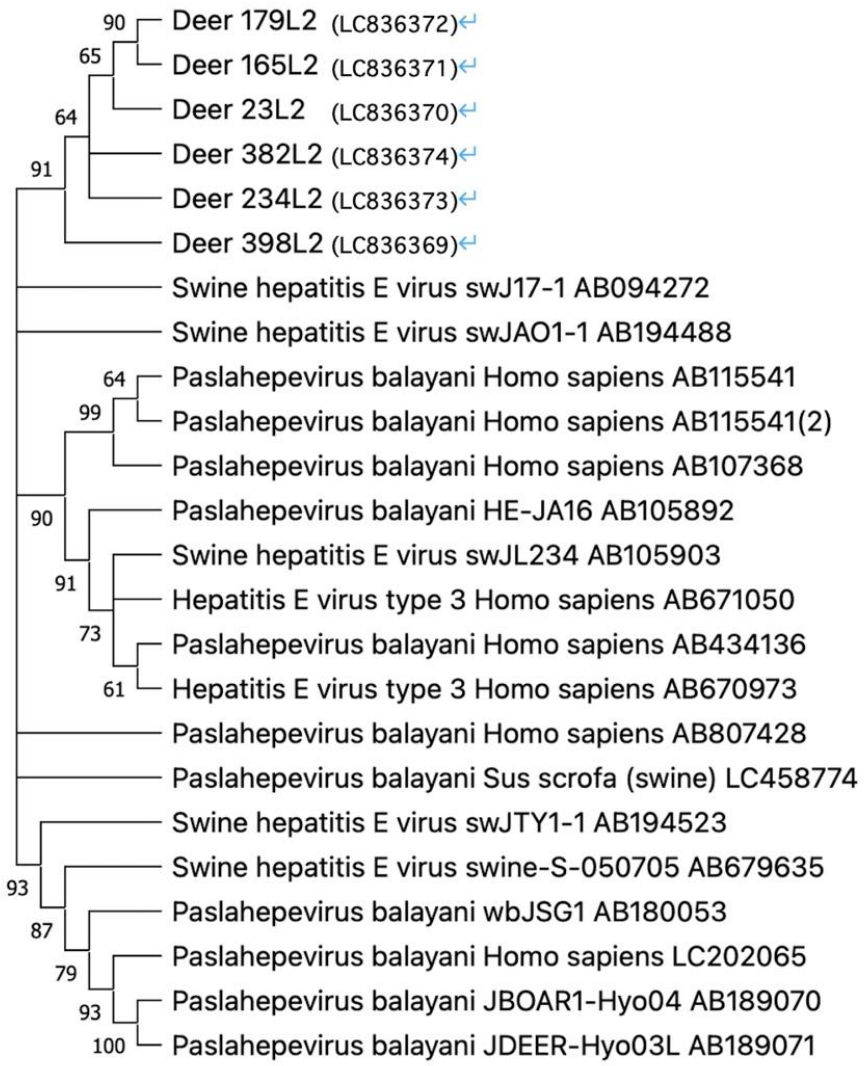


**Figure 4:** Evolutionary analysis of deer HEV by Maximum Likelihood method.

## Conclusions

The number of human-reported cases of hepatitis E in Hokkaido by the National Institute of Infectious Diseases ranges from 9.8 to 19% (National Institute of Infectious Diseases, 2024). Given that wild deer entering urban areas from mountainous forests have the opportunity to be efficiently infected with HEV, public education on infection prevention is needed as a countermeasure. We urge people to avoid careless contact with wild animals, practice good hand hygiene, and eat wild animal meat and organs only after thoroughly cooking—prompt cleanup of deer excrement in public areas. Conduct testing of captured deer to identify infected individuals. Although the direct risk of infection from HEV-infected deer entering urban areas is considered low, combining the above factors could spread localized infection. Therefore, residents and communities should consider preparing for an early response.

**Article Information**

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Authors contribution. Y.K., K.H. Sample collection, preliminary processing, investigation, molecular biology analysis, writing - original draft, data curation, software application; K.H: conceptualization, methodology, validation, supervision, writing - review and editing, project administration, funding acquisition.

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