



NOTE

Wildlife Science

Encephalitis induced by a newly discovered ruminant rhadinovirus in a free-living Formosan sambar deer (*Rusa unicolor swinhoei*)

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ABSTRACT. We documented a case of a free-living Formosan sambar deer (*Rusa unicolor swinhoei*) infected with a newly discovered ruminant Rhadinovirus (RuRv). Non-purulent encephalitis was the primary histological lesion of the sambar deer. We conducted nested PCR to screen for herpesvirus using generic primers targeting the *DNA polymerase gene*. In addition, we found that *DNA polymerase gene* of the sambar deer RuRv was present in the macrophage distributed in the Virchow Robin space with histopathologic lesions by chromogenic in-situ hybridization (CISH). The phylogenetic analysis indicated a high similarity between the viral sequence isolated from fallow deer and our case. This result suggests the possibility of cross-species transmission from other exotic Cervidae reservoir to the Formosan sambar deer.

KEY WORDS: cervidae, Formosan sambar deer, ruminant rhadinovirus

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The Formosan sambar deer (*Rusa unicolor swinhoei*) is an endemic subspecies which is mainly found in the natural forests of the Central Mountain Range of Taiwan [14]. It is listed as a rare and valuable species under the Wildlife Conservation Act [3]. We here describe a case of ruminant Rhadinovirus (RuRv) infection in a wild Formosan sambar deer.

RuRv is a subgroup of the genus *Rhadinovirus* which belongs to the subfamily Gammaherpesvirinae [4]. Many novel viruses discovered from diverse mammal species are Gammaherpesvirinae [5]. Malignant catarrhal fever (MCF) is an ungulate disease caused by Macavirus. Macavirus is closely related to RuRv and was previously classified as a type 1 RuRv [4]. Each virus causing MCF is maintained in the population of specific reservoirs [7], e.g., blue wildebeest (*Connochaetes taurinus*) for Alcelaphine herpesvirus 1 (AIHV-1) [10], or domestic sheep (*Ovis aries*) for ovine herpesvirus 2 (OvHV-2) [1]. Once the viruses spillover from asymptomatic reservoirs into susceptible species, they can induce MCF, a fatal lymphoproliferative disease [11]. Despite the relatedness between MCF Macavirus and RuRv, RuRv has not previously been reported as an etiological agent [4].

On 10th June 2016, a free-living adult male Formosan sambar deer was found in a recumbent position on a roadside in the Meishan district, Yushan National Park (latitude: 23.283640; longitude: 120.902723) (Fig. 1). The individual was in a very weak condition and died during transportation to the Pingtung Rescue Center for Endangered Wild Animals at Pingtung University of Science and Technology. We conducted a necropsy and collected tissue samples for further molecular and histopathologic examinations. The gross examination showed that several abrasive skin lesions were scattered around the body and velvet antlers. We also found diffuse lung edema and hemorrhage. The histopathological examination revealed lymphocytic perivascular cuffing, perivascular space edema, and endothelial cells swelling in the cerebrum (Fig. 2A). The homogenized tissues of lung and spleen were used for virus isolation in a flask containing MDBK (Madin-Darby Bovine Kidney) cells with DMEM media. The MDBK cells grow on 75T flask at 37°C for virus isolation, blind passages in MDBK cell were made. After the second passage, the cell showed cell rounding, the herpesvirus characteristic cytopathic effect and different from the uninfected control cell. For

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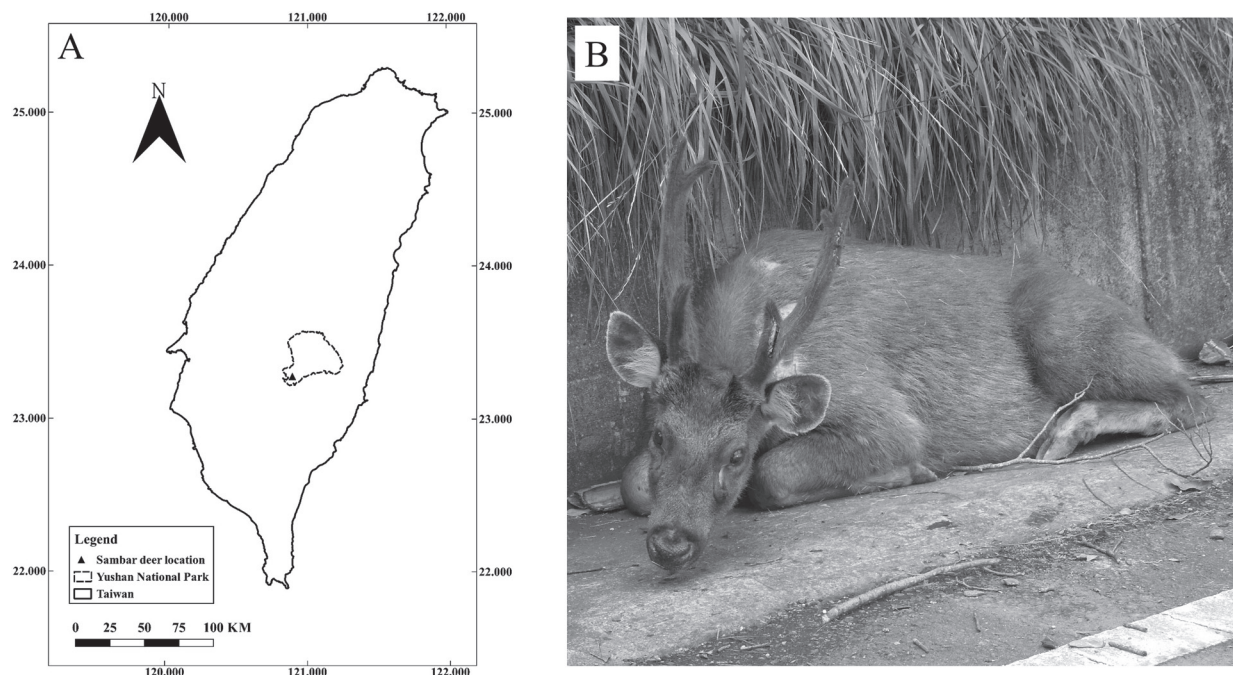


Fig. 1. The location and appearance of the Formosan sambar deer (*Rusa unicolor swinhoei*) found with sambar deer Rhadinovirus infection in Yushan National Park, Taiwan. (A) The individual was found in the Meishan district in the southeastern part of Yushan National Park. (B) The individual was weak, emaciated and sternally recumbent when found, had multiple skin abrasions, and displayed no response to humans approaching.

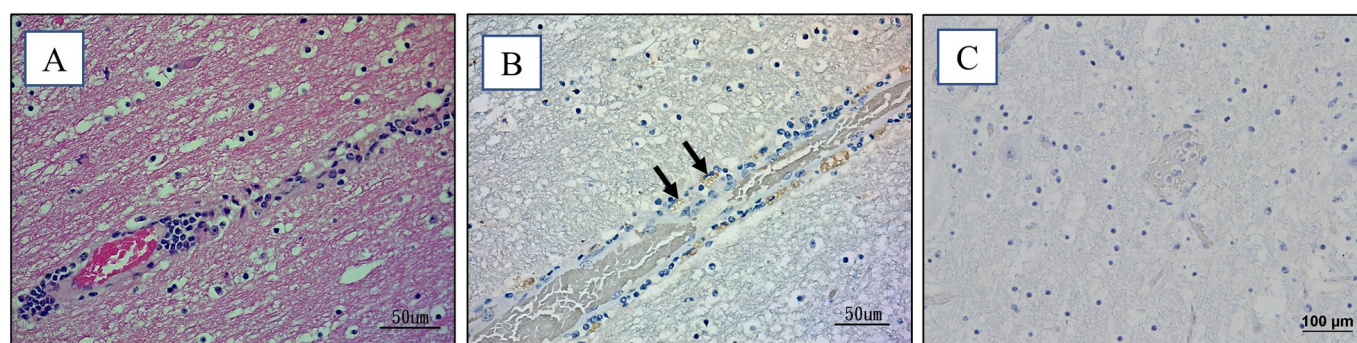


Fig. 2. Histopathology of the cerebrum of the infected individual (Fig. 1) showed lymphocytic perivascular cuffing and edema of the perivascular space (A; H&E). The distinct dark brown signal of chromogenic *in-situ* hybridization indicated the presence of the specific DNA sequence in macrophage around the perivascular cuffing lesion (B; CISH). No signal in negative control (C; CISH).

each passage, we collected cell and supernatant for real-time PCR. The real-time PCR was carried out on the CFX96 Connect™ detection system (Bio-Rad, Hercules, CA, U.S.A.) using the iQ SYBR® Supermix (Bio-Rad) in a final volume of 20 μ l. The condition started with 95°C for 5 min followed by 40 cycles of 95°C for 10 sec, 54°C for 20 sec and 72°C for 15 sec. We designed the primers of Deer-H-F (5'-CGCAGAAACGGTTACTCA-3') and Deer-H-R (5'-TCCGTGTCGCCGTAGATAA-3'). The primers were targeted on a portion of the herpesvirus' *DNA polymerase gene*, the product size was around 162bp. A single sharp peak was observed in the melting curve. A dilution series of herpesvirus template concentrations were also established to build the standard curve. The real-time PCR quantification showed that the virus we isolated was gradually adapted to MDBK cell line. From the first to third passage, the virus quantity increased quickly, fourth to tenth passage it remained the same virus level. From hereupon, we call these samples "isolated herpesvirus" for ease of understanding.

We performed a nested polymerase chain reaction (nested PCR) screening with degenerative generic primers for herpesvirus on the collected tissues and isolated herpesvirus. The primers designed by VanDevanter *et al.* (1996) were targeted on a portion of the herpesvirus' *DNA polymerase gene*. The first nested PCR contained two upstream primers (DFA: 5'-GAYTTYGCNAGYYTNTAYCC-3'; and ILK: 5'-TCCTGGACAAGCAGCARNYSGCNMTNAA-3') and one downstream primer (KG1: 5'-GTCTTGCTCACCAGNTCNACNCCYTT-3'). The second nested PCR

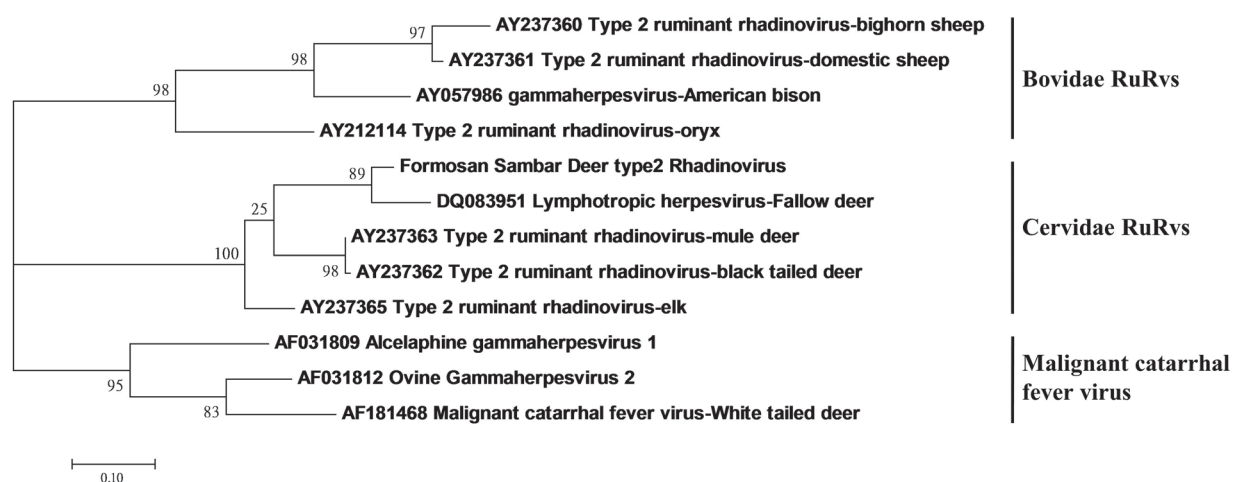


Fig. 3. Molecular phylogenetic relationship of the partial Rhadinovirus DNA polymerase gene of the Formosan sambar deer and other RuRvs sequences accessed from GenBank. The phylogenetic tree was constructed with the maximum likelihood method. The bootstrap value is shown next to the node with 1,000 replicates. The GenBank accession numbers, virus strains and animal are indicated.

used one upstream TGV (5'-TGTAACCTCGGTGTAYGGNTTYACNGGNGT-3') and downstream primer IYG (5'-CACAGAGTCCGTRTCNCCRTADAT-3'). Tissue samples from the lung, spleen, cerebrum, liver, kidney, and large intestine which had been collected during the necropsy were selected for the herpesvirus PCR screening. The tissues were first homogenized, and then the total DNA was extracted using the DNeasy blood and tissue kit (Qiagen, Valencia, CA, U.S.A.) following the manufacturer's recommended procedure. The conditions of our nested PCR amplification followed the instructions of [13] with minor modifications. Briefly, viral templates were amplified in 20 μ l reaction mixture which contained PCR buffer (1.5 mM MgCl₂, each deoxynucleoside triphosphate at a concentration of 200 μ M, Hot-StarTaq Master Mix [Qiagen]), 0.2 μ M of each PCR primer, and 2 μ l of the DNA templates. Amplification consisted of 15 min at 95°C and 35 cycles of 30 sec at 94°C, 30 sec at 47°C, and 60 sec at 72°C, with a final extension of 7 min at 72°C. The second nested PCR was performed under the same conditions used during the first reaction. The samples from the spleen, lung, large intestine, and viral culture tested positive for PCR with the expected size of 228 bp of PCR amplicons. We also conducted the orbivirus RT-PCR screening with generic primers based on the conditions described by [8]. However, the orbivirus RT-PCR screening for the collected tissues and viral culture all tested negative.

The PCR amplicons were sequenced in an ABI377 sequencer using the ABI PRISM dye-terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase (Perkin-Elmer, Applied Biosystems, Foster City, CA, U.S.A.). All the sequences of PCR amplicons from the sampled tissues and the isolated herpesvirus were identical. The sequence was registered in the NCBI GenBank with accession number MF286530. Using this sequence, a BLAST search was conducted against GeneBank (BLAST; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) with the database and algorithm of Nucleotide collection (nt/nr) and blastn, respectively. The BLAST search resulted in a match between our isolated herpesvirus and other RuRv, indicating that our isolated sambar deer herpesvirus is a novel RuRv which is phylogenetically closely related to the lymphotropic herpesvirus of the fallow deer (access no. DQ083951). We aligned the nucleotide sequences with the software *MEGA* (Molecular Evolutionary Genetics Analysis) version 6, using the Clustal W multiple alignment function [12]. We then used the maximum likelihood method based on the Kimura 2-parameter model with 1,000 bootstrapping replications to construct the phylogenetic relationships among the different RuRvs [6]. Figure 3 demonstrates that the isolated sambar deer herpesvirus is located in the subclade of the Cervidae-originated RuRvs.

We designed a specific oligonucleotide probe labeled with digoxigenin at the 3' end to detect the *DNA polymerase gene* of the sambar deer RuRv in the tissue samples. The probe sequence was 5'-ACCTCACACTGGCAGACATATCC-3'. We compared the sequence of our oligonucleotide probe with known sequences in the GenBank database using the BLAST search to ensure our probe's specificity. The designed probe sequence was 100% complementary only to one other published RuRv, a fallow deer lymphotropic herpesvirus (access no. DQ083951), but not to other closely related lymphotropic herpesviruses. We then conducted a chromogenic in-situ hybridization (CISH) using a commercial kit (TASH01D, BioTnA Inc., Kaohsiung, Taiwan) following the manufacturer's recommended procedures to detect the presence of RuRv DNA in the cerebrum tissue. Briefly, after deparaffinization in xylene and ethanol, we incubated the slides in de-peroxidase reagent at room temperature for 10 min to inactivate intrinsic peroxidase. Thereafter, we applied a 50 μ l of 1:100 probe to the tissue, covered with a coverslip, and placed it on a hot plate at 80°C for 5 min. The slide was immediately transferred into a humidity chamber and hybridized for 16 hr at 37°C. Probe detection was performed with a sequential incubation of an anti-dig and anti-mouse-HRP antibody for one night and 30 min at room temperature, respectively. Finally, the slide was counterstained with haematoxylin. We used the herpesvirus' negative tissues screened by nested-PCR from another sambar deer as the negative CISH control. The sambar deer RuRv was identified by a distinct dark brown signal within the cells. We thus found that the sambar deer RuRv was present in the macrophage distributed in

the Virchow Robin space (Fig. 2B). The negative CISH control using tissues from another sambar deer died due to other causes did not return a signal (Fig. 2C).

Consequently, we present a newly discovered case of a free-living Formosan sambar deer infected with a previously unknown strain of RuRV. The mild lymphocytic perivascular cuffing in the cerebrum was the primary lesion associated with the Rhadinovirus we isolated. However, we were not able to detect the virus sequence based on the PCR in the cerebrum. This might be caused by the limitation of virus distribution in the cerebrum. In addition, due to the lack of background information about the sambar deer, we are not able to rule out the possibility that other factors might have been responsible for the deer becoming more susceptible to the RuRV infection. Such other factors could have played a role because no direct association has been previously documented between symptomatic disease and RuRVs.

Li *et al.* [4] screened the peripheral blood leukocytes from various domestic and free range ruminant species and found genetic similarities between the isolated RuRV and the corresponding ruminant species. Their finding indicated that a co-evolutionary relationship exists between the identified RuRVs and their hosts.

Our phylogenetic analysis indicated that our sambar deer RuRV was closely related to a fallow deer RuRV virus. However, based on the Cervidae phylogeny, sambar deer is phylogenetically closely related to other *Cervus* species, but relatively distantly related to fallow deer [9]. This distant relationship clashes with the previously documented close co-evolutionary relationship between RuRVs and their hosts (see above). Therefore, our finding indicates the possibility of cross-species transmission from other deer reservoirs to the Formosan sambar deer. Like other herpesviruses, it is possible that RuRVs could be pathogenic to certain species or under certain conditions [2]. The Formosan sambar deer is currently the only endemic Cervinae species in the region where the infected deer was discovered (Fig. 1). Therefore, perhaps the source of our newly discovered RuRV was another exotic Cervidae species. A likely candidate is the fallow deer, a domesticated deer introduced into Taiwan. However, the dying sambar deer was in a remote mountainous area; therefore, it remains unclear how this novel RuRV may have infected the sambar deer.

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REFERENCES

- Baxter, S. I. F., Pow, I., Bridgen, A. and Reid, H. W. 1993. PCR detection of the sheep-associated agent of malignant catarrhal fever. *Arch. Virol.* **132**: 145–159. [Medline] [CrossRef]
- Ensser, A., Neipel, F. and Fickenscher, H. 2002. Rhadinovirus pathogenesis, pp. 349–429. *In: Structure-Function Relationships of Human Pathogenic Viruses* (Holzenburg, A. and Bogner, E. eds.), Kluwer Academic Publishers, New York.
- Forest Bureau, Council of Agriculture, Executive Yuan. 2014. Schedule of protected wildlife. Forest Bureau, Council of Agriculture, Executive Yuan, Taipei, Taiwan. <http://www.conservation.forest.gov.tw> [accessed on August, 20].
- Li, H., Gailbreath, K., Flach, E. J., Taus, N. S., Cooley, J., Keller, J., Russell, G. C., Knowles, D. P., Haig, D. M., Oaks, J. L., Traul, D. L. and Crawford, T. B. 2005. A novel subgroup of rhadinoviruses in ruminants. *J. Gen. Virol.* **86**: 3021–3026. [Medline] [CrossRef]
- McGeoch, D. J., Gatherer, D. and Dolan, A. 2005. On phylogenetic relationships among major lineages of the Gammaherpesvirinae. *J. Gen. Virol.* **86**: 307–316. [Medline] [CrossRef]
- Nei, M. and Kumar, S. 2000. *Molecular Evolution and Phylogenetics*. Oxford University Press, New York.
- O'Toole, D. and Li, H. 2014. The pathology of malignant catarrhal fever, with an emphasis on ovine herpesvirus 2. *Vet. Pathol.* **51**: 437–452. [Medline] [CrossRef]
- Palacios, G., Cowled, C., Bussetti, A. V., Savji, N., Weir, R., Wick, I., Travassos da Rosa, A., Calisher, C. H., Tesh, R. B., Boyle, D. and Lipkin, W. I. 2011. Rapid molecular strategy for orbivirus detection and characterization. *J. Clin. Microbiol.* **49**: 2314–2317. [Medline] [CrossRef]
- Pitra, C., Fickel, J., Meijaard, E. and Groves, P. C. 2004. Evolution and phylogeny of old world deer. *Mol. Phylogenet. Evol.* **33**: 880–895. [Medline] [CrossRef]
- Plowright, W., Ferris, R. D. and Scott, G. R. 1960. Blue wildebeest and the aetiological agent of bovine malignant catarrhal fever. *Nature* **188**: 1167–1169. [Medline] [CrossRef]
- Russell, G. C., Stewart, J. P. and Haig, D. M. 2009. Malignant catarrhal fever: a review. *Vet. J.* **179**: 324–335. [Medline] [CrossRef]
- Tamura, K., Stecher, G., Peterson, D., Filipinski, A. and Kumar, S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **30**: 2725–2729. [Medline] [CrossRef]
- VanDevanter, D. R., Warrenner, P., Bennett, L., Schultz, E. R., Coulter, S., Garber, R. L. and Rose, T. M. 1996. Detection and analysis of diverse herpesviral species by consensus primer PCR. *J. Clin. Microbiol.* **34**: 1666–1671. [Medline]
- Yen, S.C., Wang, Y. and Ou, H.Y. 2014. Habitat of the vulnerable Formosan sambar deer *Rusa unicolor swinhoii* in Taiwan. *Oryx* **48**: 232–240. [CrossRef]