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Original Article

Occurrence of oral shedding of retroperitoneal fibromatosis-associated herpesvirus and lymphocryptovirus in free-ranging macaques, Thailand

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Abstract

Oropharyngeal swabs of 262 free-ranging macaques (*Macaca fasicularis* and *M. mulatta*) were tested for *Herpesviridae*. The occurrence was 24.4% (64/262), and no macaques showed clinical signs of the disease. The virus was restricted to the northern and north-eastern regions of Thailand. DNA polymerase genes of the 64 positive samples were then characterized and analyzed. Eight samples (3.1%) were found to be closely related to retroperitoneal fibromatosis-associated herpesvirus (RFHV) and were classified in the genus *Rhadinovirus*, subfamily *Gammaherpesvirinae*. The phylogram of the virus revealed it to be species-specific within the macaque population. A total of 56 samples (21.4%) exhibited a nucleotide relationship with lymphocryptovirus (LCV) in the genus *Lymphocryptovirus*, subfamily *Gammaherpesvirinae*. The gender of the macaques did not significantly correlate with gammaherpesvirus infection. Our results indicate that LCV is endemic in the macaque population in Thailand, but RFHV is not endemic.

Keywords: Gammaherpesvirus, Free-ranging macaque, Lymphocryptovirus, Retroperitoneal fibromatosis-associated herpesvirus

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Introduction

Retroperitoneal fibromatosis-associated herpesvirus (RFHV) and lymphocryptovirus (LCV), a member of the subfamily *Gammaherpesvirinae*, are assigned to the genera *Rhadinovirus* and *Lymphocryptovirus*, respectively (Davison, 2010). RFHV and LCV have been frequently detected in several species of macaques, including *Macaca nemestrina*, *M. mulatta*, *M. fascicularis*, and *M. fuscata* (Auerbach et al., 2000; Davison et al., 2009; Giddens et al., 1985; Rose et al., 1997; Simmons, 2010; White et al., 2009). RFHV infection is generally subclinical in healthy natural hosts, and overt disease is thought to arise only when hosts are immune suppressed (Giddens et al., 1985; Simmons, 2010). RHFV causes retroperitoneal fibromatosis (RF), which was initially recognized as a disease in 1976 (Giddens et al., 1985). RF is characterized by the aggressive proliferation of highly vascular, fibrous tissue subjacent to the peritoneum covering the ileocecal junction, associated mesenteric lymph nodes, and the gastrointestinal tract. The disease characteristics are similar to Kaposi's sarcoma, which is caused by human herpesvirus 8 (Giddens et al., 1985; Rose et al., 1997). More than 90% of adult macaques have persistent LCV infection, which is generally not associated with disease (Fahey and Westmoreland, 2012). Immunologically normal macaques do not exhibit clinical signs, but in immune deficient macaques, the virus can induce epithelial cell lesions resembling oral hairy leukoplakia in human herpesvirus 4 (Epstein-Barr virus)-infected AIDS patients (Kutok et al., 2004; Sasseville and Mansfield, 2010; Wachtman and Mansfield, 2008). While there have been some reports of RFHV and LCV infection in captive macaques, the diversity of these infectious agents in free-ranging macaques is limited (Auerbach et al., 2000; Davison et al., 2009; Fahey and Westmoreland, 2012; Giddens et al., 1985; Rose et al., 1997; Simmons, 2010; White et al., 2009). The present study reports estimates of the occurrence of oral shedding of LCV and RFHV in free-ranging macaques (*M. mulatta* and *M. fascicularis*) in Thailand. The results reveal that LCV is more endemic in macaque populations than rhadinovirus, indicating that LCV circulates well within the population.

Materials and Methods

Genomic DNA extraction: A total of 262-DNA samples were extracted from the oropharyngeal swabs of 227 (male = 154, female=73) long-tailed macaques (*M. fasicularis*) and 35 (male=18, female=17) rhesus macaques (*M. mulatta*) using a viral nucleic acid extraction kit II (Geneaid, Taiwan). The macaques were captured from temples, parks and schools in eight provinces in the northern, north-eastern, southern, central, and western regions of Thailand from December 2018 to March 2019 (Fig. 1). The samples used in this study were approved by the Animal Care and Use Committee of the Faculty of Veterinary Science, Mahidol University (Protocol No. MUVS-2018-09-49).

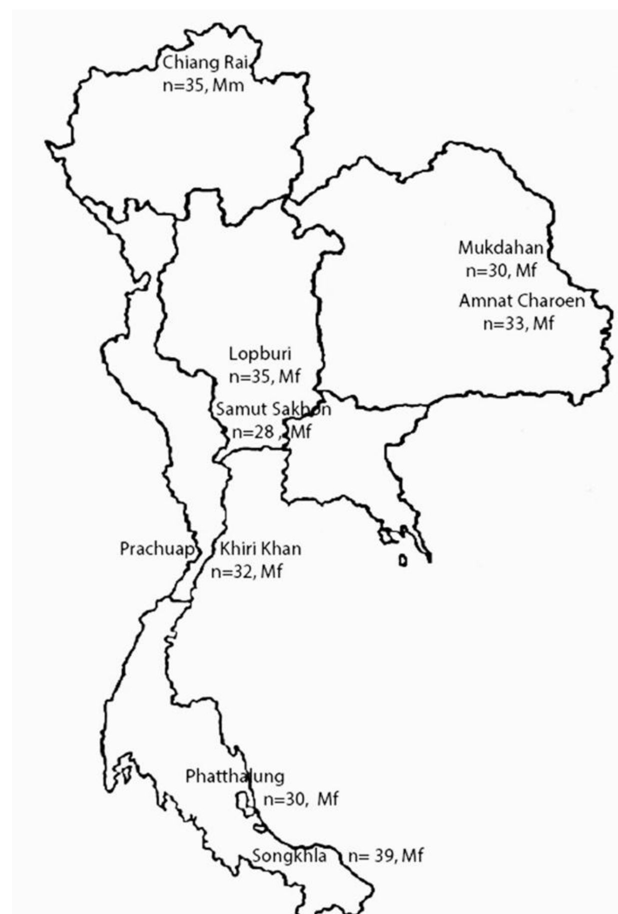


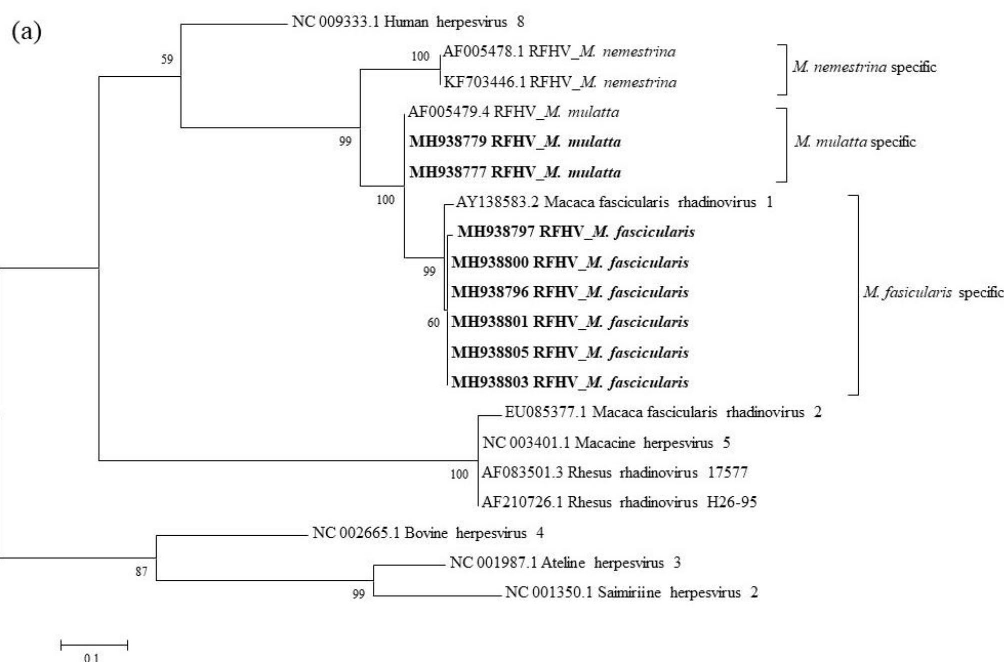
Figure 1 Geographical map showing sampling sites of macaques (*M. fasicularis*: Mf and *M. mulatta*: Mm) in Thailand.

***Herpesviridae* detection:** Primers DFA (5' GAY TTY GCN AGY YTN TAY CC 3'), IL (5 'TCC TGG ACA AGC ARN YSG CNM TNA A 3'), and KGI (5' GTC TTG CTC ACC AGN TCN ACN CCY TT 3') were used for the first PCR and primers TGV (5' TGT AAC TCG GTG TAY GGN TTY ACN GGN GT 3') and IYG (5' CAC AGA GTC CGT RTC NCC RTA DAT 3') were used for the second PCR, resulting in a 207-bp PCR product (VanDevanter et al., 1996). These primers are specific to the DNA polymerase gene of the *Herpesviridae* family. The PCR mixture contained 3 µl of template DNA, 2.5 µl of 10× PCR buffer with 20 mM $MgCl_2$, 1 mM of dNTPs, 2.5 units of i-*Taq* DNA polymerase (iNtRON, Korea), and 0.5 µM of each primer. Sterile nuclease-free water was added to increase the volume of the mixture to 25 µl. The PCR reaction was performed under the following conditions: 2 min at 94ºC for initial denaturing, followed by 35 cycles of 45 s at 94ºC, 45 s at 50ºC, and 30 s at 72ºC. The reaction was terminated with a final cycle of 72ºC for 7 min. The components for the second PCR were the same as those for the first PCR, with the exception of the primers. The PCR reaction was performed as described above with an annealing temperature of 56 ºC.

Phylogenetic tree analysis: Positive samples from the nested PCR were directly sequenced by Macrogen Inc. (Seoul, South Korea) using the Sanger method. Sequencing data was analyzed using the Basic Local Alignment Search Tool (BLAST). A phylogenetic tree was constructed from 112 nucleotide sequences of DNA polymerase genes, using the maximum likelihood method based on the Kimura two-parameter model, with a bootstrap value of 1,000 replicates, in the MEGA7 version 7.0 software (Kimura, 1980; Kumar et al., 2016).

Results

Oropharyngeal swabs of macaques (*M. fascicularis* and *M. mulatta*) from eight provinces of Thailand were tested for *Herpesvidae* by nested PCR. Of the 262 macaques tested, 64 (24.4%) were positive for *Herpesviridae*. At the time of sample collection, the macaques showed no clinical signs of disease. In Table 1, the virus was mostly distributed in northern and north-eastern regions of Thailand with 32.8% (21/64) and 29.7% (19/64), respectively. The positive rate of gammaherpesvirus in male (23.8%) and female (25.6%) macaques indicates no difference in terms of gender. The positive samples were further sequenced and compared to the sequences deposited in GenBank. BLAST searches showed that the DNA polymerase sequences obtained in this study belong to the subfamily *Gammaherpesvirinae* (Accession no. MH938751-MH938806). A phylogenetic tree was subsequently constructed, revealing that the DNA polymerase gene of eight (3.1%) macaques is closely related to RFHV in the genus *Rhadinovirus*, whereas the DNA polymerase gene of 56 (21.4%) macaques exhibited a nucleotide relationship with LCV in the genus *Lymphocrypovirus* (Fig. 2). The nucleotide sequence of DNA polymerase-RFHV obtained in this study was similar to that of RFHV isolate YN91 (Accession no. AF005479.4), with 94.31 to 100% identity. The deduced amino acid of this gene was related to *Macaca fascicularis* rhadinovirus 1 (Accession no. AAN35122.2) with 90.24 to 100% identity. The DNA polymerase–LCV in this study showed 96.45 to 100% nucleotide identity with *Macaca fascicularis* lymphocryptovirus isolate 1698 (Accession no. JQ062969.1) and 97.87 to 100% amino acid identity with *Macaca fascicularis* lymphocryptovirus (Accession no. AFK84418.1). The phylogram in Fig. 2a shows that the RFHV-DNA polymerase sequences detected in *M. fasicularis* and *M. mulatta* are grouped into different clusters with statistically different clusters (bootstrap value = 100%). For the lymphocryptovirus phylogram, DNA polymerase sequences obtained in this study are closely related to lymphocryptovirus of Old World monkeys and apes, including human herpesvirus 4. The New World monkey virus lineage forms a sister clade to that of the Old World monkey and ape viruses (Fig. 2b).



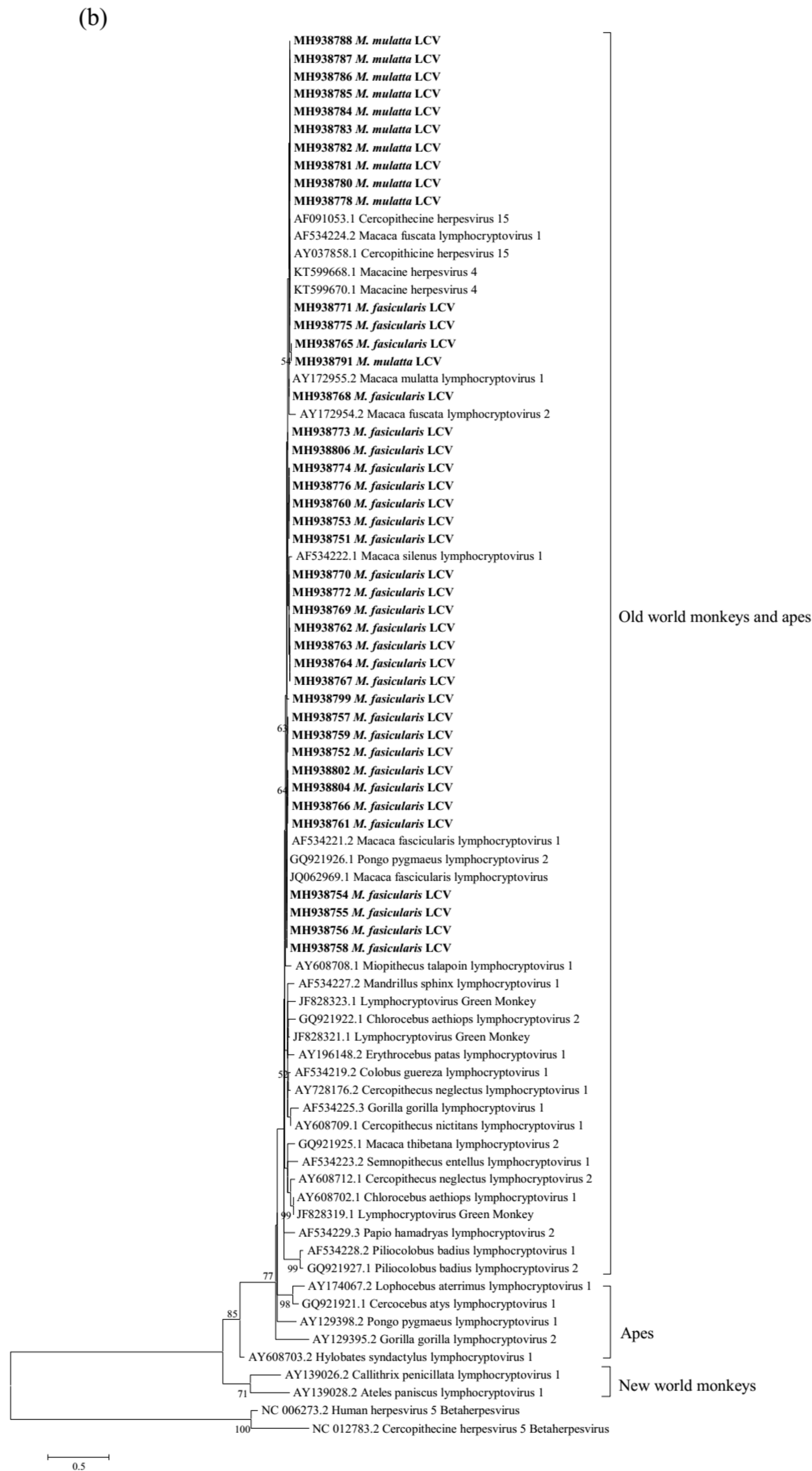


Figure 2 Phylogenetic tree resulting from analysis of selected 112-bp fragments of the herpesvirus DNA polymerase gene. (a): Rhadinovirus phylogram. (b): Lyphocryptovirus phylogram. The tree is drawn to scale, with branch lengths measured by the number of substitutions per site. Numbers at each node indicate the bootstrap percentage. The sequences analyzed in this study are shown in bold.

Table 1 Sample details and numbers of positives for *Herpesviridae*.

Location		Monkey species	Number of monkeys			Number of LCV positives (%)			Number of RFHV positives (%)			Number of *Herpesviridae* positives (%)		
			Male	Female	Total	Male	Female	Total	Male	Female	Total	Male	Female	Total
Northern region	Chiang Rai Province	*M. mulatta*	18	17	35	7	12	19	1	1	2	8	13	21
North-eastern region	Amnat Charoen Province	*M. fascicularis*	27	6	33	13	1	14	0	0	0	13	1	14
	Mukdahan Province	*M. fascicularis*	26	4	30	4	1	5	0	0	0	4	1	5
Western region	Prachuap Khiri Khan Province	*M. fascicularis*	10	22	32	2	1	3	0	0	0	2	1	3
Central region	Samut Sakhon Province	*M. fascicularis*	19	9	28	2	1	3	0	0	0	2	1	3
	Lopburi Province	*M. fascicularis*	16	19	35	3	4	7	0	0	0	3	4	7
Southern region	Phatthalung Province	*M. fascicularis*	27	3	30	0	0	0	0	0	0	0	0	0
	Songkhla Province	*M. fascicularis*	29	10	39	4	1	5	5	1	6	9	2	11
	Total		172	90	262	35	21	56 (21.4)	6	2	8 (3.1)	41	23	64 (24.4)

Discussion

In this study, oropharyngeal swabs of 262 free-ranging macaques (*M. fasicularis* and *M. mulatta*) were tested for *Herpesviridae* and found 24.4% (64/262) were positive. After DNA polymerase genes analysis, eight samples (3.1%) were RFHV and 56 samples (21.4%) were LCV. The incidence rate of RFHV that has been reported is approximately 1% in all age groups and 5-10% in colony-born juvenile macaques (Giddens et al., 1985; White et al., 2009). However, breeding colonies of *M. mulatta* were previously reported to have RFHV infection prevalence up to 38.9% (35/90) in saliva samples (White et al., 2009). In Thailand, antibodies against gammaherpesvirus have been found in approximately 96.4% (241/250) of free-ranging *M. fasicularis* (Ishida and Varavudhi, 1992). These findings suggest that gammaherpesvirus, especially LCV, is endemic among the macaque population.

The result shows that the RFHV-DNA polymerase sequences detected in *M. fasicularis* and *M. mulatta* are grouped into different clusters with statistically different clusters (bootstrap value = 100%) (Fig. 2a). This provides evidence that the virus is species specific. A similar result was seen for macaque rhadinovirus 2 glycoprotein B sequence analysis of *M. mulatta*, *M. fasicularis* and *M. nemestrina*, providing evidence that the virus is species specific for macaques (Auerbach et al., 2000). Moreover, the RFHV-DNA polymerase sequences are closer to the human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus: KSFV)-DNA polymerase sequence than to any other rhadinovirus (Fig. 2a). Whole genome sequencing of the RFHV detected from *M. nemestrina* was performed, and showed the RFHV sequence reveals several similarities to KSHV, including unique genes that play critical roles in the biology and pathology of the virus (Bruce et al., 2013). Thus, RFHV infection in macaques may be an appropriate animal model for KSHV infection in humans.

Based on DNA polymerase and glycoprotein B amino acid sequences, lymphocryptoviruses are grouped in three major clades, designated A, B, and C. Clade A contains lymphocryptovirus detected in New World monkeys. Clade B contains lymphocryptovirus detected in Old World monkeys and some apes, including human herpesvirus 4. Clade C contains only lymphocryptovirus detected in apes (Ehlers et al., 2010). The lymphocryptoviruses in this study are grouped in Clade B. However, the lymphocryptovirus sequences of this group show short branch lengths, resulting in limited detail for comparison (Fig. 2b).

In conclusion, our results confirm that lymphocryptovirus infection is common in colonies of rhesus macaques and long-tailed macaques, while rhadinovirus infection is rare.

Acknowledgements

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