Short Communication

Detection of elephant endotheliotropic herpesvirus 4 in captive asian elephants (*Elephas maximus*) in Thailand

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Abstract

Elephant endotheliotropic herpesviruses (EEHVs) can cause fatal hemorrhagic disease in elephants, especially young captive Asian elephants (*Elephas maximus*). Currently, seven EEHV types have been reported. In this study, EEHVs were examined in whole-blood samples derived from 56 captive Asian elephants from eight provinces in Thailand by nested PCR using primers specific to the viral DNA polymerase gene in an attempt to monitor EEHV elephant cases. After EEHV testing, one sample (1.78%) was positive and found to be closely related to EEHV4 with 99% amino acid identity. This sample was from a three-year-old female Asian elephant with no clinical signs. These data suggest that asymptomatic EEHV4 infection can occur in Asian elephants.

Keywords: Asian elephant, *Elephas maximus*, EEHV4, elephant endotheliotropic herpesvirus

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Introduction

Elephant endotheliotropic herpesvirus (EEHV) is a member of the *Proboscivirus* genus. The virus is divided into seven groups, namely, EEHV1 to EEHV7 (Davison et al., 2009; Garner et al., 2009; Latimer et al., 2011; Richman et al., 1999; Zong et al., 2015). EEHVs cause lethal hemorrhagic disease in both Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephants, but appear to be more pathogenic in Asian elephants. The majority of cases of fatal hemorrhagic disease in young Asian elephants are caused by EEHV1, EEHV3, EEHV4, and EEHV5 (Bouchard et al., 2014; Sripiboon et al., 2013; Wilkie et al., 2014; Zachariah et al., 2013; Zong et al., 2014). However, EEHV1 and EEHV5 have been reported in healthy Asian elephants (Atkins et al., 2013; Sariya et al., 2012; Stanton et al., 2014), and EEHV2, EEHV3, EEHV6, and EEHV7 have been detected in healthy African elephants (Zong et al., 2015). Multiple EEHV infections have been reported in both Asian and African individual elephants, suggesting that infection with one subtype of EEHV does not prevent them from infection with other subtypes of EEHVs (Stanton et al., 2013; Zong et al., 2015).

Because EEHVs cannot be cultured, polymerase chain reaction (PCR) or real-time PCR-based methods have been used to detect the virus in whole-blood samples, samples of necropsied tissue, conjunctiva swabs, trunk-wash fluid, trunk swabs, or palate samples (Hardman et al., 2012; Latimer et al., 2011; Sariya et al., 2012; Stanton et al., 2014). In the present study, EEHVs were examined in whole-blood samples to monitor susceptible elephants and provide knowledge about status of the disease in Asian elephants in Thailand.

Materials and Methods

Sample collection and genomic DNA extraction: In 2009, whole-blood samples were collected from 56 captive Asian elephants from eight provinces in Thailand. Prior to blood collection, the elephants were controlled by elephant keeper. Ten milliliters of whole blood from the ear vein of each elephant was collected in an EDTA blood collection tube (BD Diagnostic Systems). The practices with elephants in this study were approved by the Animal Care and Use Committee of the Faculty of Veterinary Science, Mahidol University (Protocol no. MUVS 2009-05). Genomic DNA was extracted from the whole-blood samples using Phenol/Chloroform/Isoamyl alcohol. Briefly, 400 µl of lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl, 10% SDS, pH 7.5) was added to a tube containing 100 µl of whole blood and then Proteinase K was added to the tube for a final concentration of 200 µg. The tube was incubated at 56 ^{o}C for 4 hours and then 500 µl of a Phenol/Chloroform/Isoamyl alcohol solution (25: 24: 1) was added to the tube. The tube was mixed by vortex and centrifuged at 10,000 ×g for 10 minutes. The aqueous solution on top was placed into a new tube, and an equal volume of the Phenol/Chloroform/Isoamyl alcohol solution was added to this tube. The tube was mixed and centrifuged as described above. Next, 1/10 volume of 3M sodium acetate and 2 volume of absolute alcohol were added to the precipitated DNA. The tube was incubated at -20 ^{o}C for 30 minutes and centrifuged at 10,000 ×g for 30 minutes at 4 ^{o}C. The DNA pellet was washed twice with 500 µl of 70% ethyl alcohol and then air-dried. The DNA pellet was resuspended with 100 µl of nuclease-free water. The DNA samples were kept at -20 ^{o}C until use.

β-actin detection by real-time PCR: Before detection of EEHVs, the samples were tested for presence of the β-actin housekeeping gene to confirm the quality of the DNA. Primers β-actin-F (5'-CGG GAC CTG ACT GAC TAC CTC-3') and β-actin-R (5'-CCT TAA TGT CAC GCA CGA TTT CC-3') were used to generate a PCR product with a size of 94 bps (Sariya et al., 2012). The real-time SYBR Green I-based PCR mixture contained 2 µl of template DNA, 12.5 µl of 2× QuantiTect SYBR Green PCR (Qaigen), and 0.3 µM each of the forward and reverse primers. Sterile, nuclease-free water was added to bring the mixture to 25 µl. The reactions were carried out in the Chromo4 real-time PCR instrument (BioRad) under the following conditions: 15 minutes at 95 ^{o}C for initial denaturation, followed by 40 cycles of 15 seconds at 94 ^{o}C, 30 seconds at 56 ^{o}C, and 30 seconds at 72 ^{o}C. After PCR cycling, melting-curve analysis was performed on the amplified PCR product at 50-95 ^{o}C by increasing the temperature by 0.2 ^{o}C/3 sec with continuous fluorescence measurement.

EEHV detection by nested PCR: Primers specific to the DNA polymerase gene listed in Table 1 (Latimer et al., 2011; Zong et al., 2015). Nested PCR was performed in a total volume of 25 µl. For the first PCR, the reaction mixture contained 12.5 µl of 2 µl of template DNA, 2.5 µl of 10× Mg^{+2} free buffer, 1.5 mM of Mg^{+2} solution, 1 mM of dNTPs, 2.5 units of i-*Taq* DNA polymerase (iNtRON), and 0.5 µM each of the forward and reverse primers. Sterile, nuclease-free water was added up to 25 µl. The PCR mixture for the second PCR was the same as that used for the first PCR. The PCR parameters for the first and second rounds of PCR are demonstrated in Table 1. For the positive sample, DNA polymerase gene was amplified for a longer product a total of 1,749 bps and confirmed by the di-deoxy dye terminator DNA sequencing method. Deduced amino acid sequence (583 amino acids) was analyzed by the Basic Local Alignment Search Tool (BLAST) program. A phylogenetic tree was constructed from the deduced amino acid sequences (198 amino acids) of the DNA polymerase using the method based on the JTT matrix-based model with a bootstrap value based on 1,000 replicates (MEGA6 version 6.0 software).

Results and Discussion

Asian elephant is listed as an endangered species by the International Union for Conservation of Nature. EEHVs are a critical matter of concern in Asian elephants because infected animals have a mortality rate of up to 85% (Latimer et al., 2011). In this study, 56 samples were tested for EEHV1 to EEHV7. Of the 56 samples, one sample (1.78%) (Elephant no. 12) was positive for primers specific to the viral DNA polymerase gene for EEHV3 or EEHV4. This sample

was from a three-year-old female elephant from Tak province. After sequencing and analysis by the BLAST program to compare the sequences with sequence databases and phylogenetic tree, the sequence of this sample (accession no. KX954609) was found to have 99% amino acid identity with EEHV4 isolate North American NAP69 (accession no. KT832477.1) and grouped in EEHV4 group (Fig. 1). During blood collection, no clinical signs of illness were observed in the EEHV4-infected elephant. Basic health check and deworming were performed every 6 months for this elephant. Result showed that this elephant had asymptomatic EEHV4 infection. The first fatal case of EEHV4 was reported in a young Asian elephant in 2009 (Garner et al., 2009). Moreover, EEHV4 was first reported in Thailand in a three-year-old male captive-born Asian elephant with hemorrhagic disease (Sripiboon et al., 2013). Furthermore, EEHV4 was found in two young captive Asian elephants in central Thailand. These elephants showed clinical signs or had lesions the viral infection (Lertwatcharasarakul et al., 2015). Previously, four healthy Asian elephants in South India were found to be positive for EEHV when testing was performed using EEHV3/4 primers (Stanton et al., 2014). However, the report did not clearly identify whether the EEHV infection was EEHV3 or EEHV4. Previous reports have shown that EEHV1 viremia can be found in both clinical and subclinical EEHV1 infection. However, DNA of EEHV1 has been detected up to 28 days before the onset of clinical signs (Stanton et al., 2013). Thus, the detection of EEHVs in whole blood may help veterinarians during the initial treatment of infected elephants before the onset of irreversible disease progression. Moreover, the detection of EEHV in blood circulation should be performed during routine heath check-up, which will be beneficial to EEHV disease control in herd level.

Table 1 List of primers specific to each EEHV subtype

EEHV specific type	Primer name	Sequences 5' to 3'	PCR product size (bp)	Thermal cycler parameters
EEHV1, 2, 4, 5	PAN-EEHV-A1	5' ACA AAC ACG CTG TCR GTR TCY CCR TA 3'	500	Denature at 94 ºC for 2 min, 35 cycles of 30 sec at 94 ºC, 30 sec at 58 ºC, and 45 sec at 72 ºC, and terminate at 72 ºC for 7 min
	PAN-EEHV-B1	5' GTA TTT GAT TTY GCN AGY YTG TAY CC 3'		
	PAN-EEHV-A1	5' ACA AAC ACG CTG TCR GTR TCY CCR TA 3'	250	Denature at 94 ºC for 2 min, 35 cycles of 15 sec at 94 ºC, 15 sec at 62 ºC, and 30 sec at 72 ºC, and terminate at 72 ºC for 7 min
	PAN-EEHV-B2	5' TGY AAY GCC GTN TAY GGA TTY ACC GG 3'		
EEHV6	PAN-EEHV-A1	5' ACA AAC ACG CTG TCR GTR TCY CCR TA 3'	500	Denature at 94 ºC for 2 min, 35 cycles of 30 sec at 94 ºC, 30 sec at 58 ºC, and 45 sec at 72 ºC, and terminate at 72 ºC for 7 min
	PAN-EEHV-B1	5' GTA TTT GAT TTY GCN AGY YTG TAY CC 3'		
	PAN-EEHV-B1	5' GTA TTT GAT TTY GCN AGY YTG TAY CC 3'	470	Denature at 94 ºC for 2 min, 35 cycles of 30 sec at 94 ºC, 30 sec at 60 ºC, and 45 sec at 72 ºC, and terminate at 72 ºC for 7 min
	EEHV6-A2	5' CAT CGA TTT TGA ACT TCT CAT GGT C 3'		
EEHV3 or 4	EEHV3/4-A1	5' CGT TGA AGG TGT CGC AGA T 3'	390	Denature at 94 ºC for 2 min, 35 cycles of 30 sec at 94 ºC, 30 sec at 55 ºC, and 45 sec at 72 ºC, and terminate at 72 ºC for 7 min
	EEHV3/4-B1	5' CAG CAT CAT CCA GGC CTA CAA C 3'		
	EEHV3/4-A1	5' CGT TGA AGG TGT CGC AGA T 3'	250	Denature at 94 ºC for 2 min, 35 cycles of 15 sec at 94 ºC, 15 sec at 64 ºC, and 30 sec at 72 ºC, and terminate at 72 ºC for 7 min
	EEHV3/4-B2	5' ATC CTG GCG CAG CTG CTG AC 3'		
EEHV5	EEHV5-A1	5' CTA CAT CTA TAC AGA ACT TTC C 3'	500	Denature at 94 ºC for 2 min, 35 cycles of 30 sec at 94 ºC, 30 sec at 50 ºC, and 45 sec at 72 ºC, and terminate at 72 ºC for 7 min
	EEHV5-B1	5' GTA CCT TAG TTA CGG ACG AGA C 3'		
	EEHV5-A1	5' CTA CAT CTA TAC AGA ACT TTC C 3'	230	Denature at 94 ºC for 2 min, 35 cycles of 15 sec at 94 ºC, 15 sec at 58 ºC, and 30 sec at 72 ºC, and terminate at 72 ºC for 7 min
	EEHV5-B2	5' CGC TGT ATA TGG ATT TAC CGG 3'		
EEHV7	R1 LGH7906B	5' GCT TCA CGA AGA CCT TGT CTA CC 3'	670	Denature at 94 ºC for 2 min, 35 cycles of 15 sec at 94 ºC, 15 sec at 60 ºC, and 30 sec at 72 ºC, and terminate at 72 ºC for 7 min
	L1 LGH7924	5' CTT CGT CAA GGC CCA CGT ACG CAC C 3'		
	R1 LGH7906B	5' GCT TCA CGA AGA CCT TGT CTA CC 3'	630	Denature at 94 ºC for 2 min, 35 cycles of 15 sec at 94 ºC, 15 sec at 56 ºC, and 30 sec at 72 ºC, and terminate at 72 ºC for 7 min
	L2 LGH7925	5' GCC GTT CGG GAG AAA CTC AAG GT 3'		

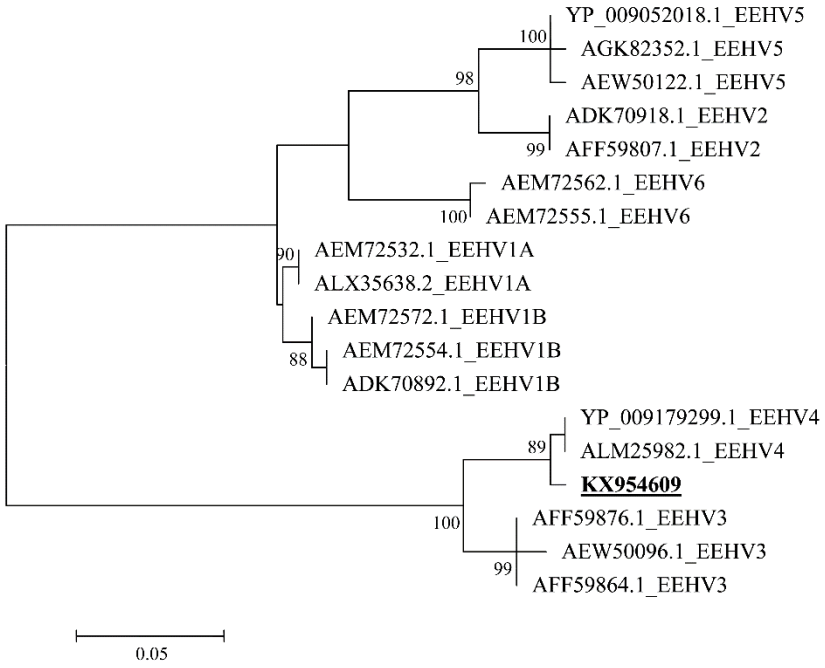

Figure 1 The DNA polymerase phylogram. A portion of deduced amino acid sequences (198 amino acids) of the DNA polymerase was used for phylogeny construction. The numbers show the percentage of times each branch was found in 1,000 bootstrap replicates. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The branches with bootstrap values lower than 85% are not shown. The sequence performed in this study is shown in underlined letters.

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บทคัดย่อ

การตรวจหาเชื้อ Elephant Endotheliotropic Herpesvirus 4 ในช้างเอเชียที่เลี้ยงในประเทศไทย

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เชื้อไวรัสเฮอร์ปีส์ในช้าง (elephant endotheliotropic herpesviruses: EEHVs) เป็นสาเหตุการตายอย่างรุนแรงในช้าง โดยเฉพาะในลูกช้างเอเชียที่เลี้ยง (*Elephas maximus*) ปัจจุบันมีรายงานการพบเชื้อไวรัสเฮอร์ปีส์ในช้างทั้งหมด 7 ชนิด การศึกษาในครั้งนี้ ทำการตรวจหาเชื้อไวรัสเฮอร์ปีส์ในเลือดช้างเอเชียทั้งหมด 56 ตัว ซึ่งเป็นช้างที่มาจาก 8 จังหวัดในประเทศไทย ด้วยเทคนิค nested PCR โดยใช้ไพรเมอร์ที่จำเพาะกับยีน DNA polymerase หลังจากที่ตรวจหาเชื้อไวรัสเฮอร์ปีส์ในตัวอย่างดังกล่าว พบว่ามี 1 ตัวอย่าง (คิดเป็นร้อยละ 1.78) ที่ให้ผลบวก โดยมีความเหมือนของลำดับกรดอะมิโนกับเชื้อ EEHV4 ถึงร้อยละ 99 ตัวอย่างนี้เป็นตัวอย่างที่ได้จากช้างเอเชียอายุ 3 ปี เพศเมีย ที่ไม่แสดงอาการของโรคไวรัสเฮอร์ปีส์ ผลการศึกษาแสดงให้เห็นว่าการติดเชื้อ EEHV4 แบบไม่แสดงอาการสามารถพบได้ในช้างเอเชีย

คำสำคัญ: *Elephas maximus* EEHV4 ช้างเอเชีย เชื้อไวรัสเฮอร์ปีส์ในช้าง

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