

Genetic Variant of Elephant Endotheliotropic Herpesvirus Detected from Captive Asian Elephants (*Elephas maximus*) in Thailand from 2007 to 2013

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Abstract

The study was aimed at characterizing elephant endotheliotropic herpesvirus (EEHV) that was detected in captive Asian elephants in Thailand from 2007 to 2013. Six tissue samples of dead elephants and two EDTA blood samples of surviving elephants in Thailand showed clinical signs or had lesions of the viral infection. Samples were extracted for DNA amplification using a PCR technique with strain specific primers based on terminase and DNA polymerase genes. Six samples gave positive amplicons for EEHV1 specific primers and two samples gave positive amplicons for EEHV3/4 specific primers. Nucleotide sequencing analysis was assured for strain identification. Five out of the six samples from EEHV1 PCR were positive for the EEHV1A strain and one sample was positive for the EEHV1B strain. The two samples of EEHV3/4 PCR positive products were revealed to be of the EEHV4 strain based on the sequencing of the partial terminase gene. Three strains of the EEHV including EEHV1A, EEHV1B and EEHV4 have been detected in Asian elephants in Thailand from 2007 to 2013. This study revealed the first EEHV1B isolate that has been detected in a captive Asian elephant in Thailand.

Keywords: Asian elephant, Elephant Endotheliotropic Herpesvirus, Terminase gene, Thailand

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Introduction

Elephant Endotheliotropic Herpesvirus (EEHV), a member of *Herpesviridae* family, causes sudden death after a 1- to 7-day onset of symptoms especially in young animals. The virus can infect both Asian elephants (*Elephas maximus*) and African elephants (*Loxodonta africana*) (Richman et al., 1999; Richman et al., 2000; Hayward, 2012). Most infected animals show clinical signs including lethargy and anorexia (Richman et al., 1999; Richman et al., 2000; Garner et al., 2009). Clinical laboratory findings are lymphopenia and thrombocytopenia. Pathological changes are edema of the neck, head, trunk and forelimbs, oral ulcerations, cyanosis of tongue and ecchymotic hemorrhage on the trunk, heart, intestine, tongue, oral mucosa, lymph node and kidneys. Basophilic intranuclear viral inclusion bodies in the capillary endothelial cells of the heart, lung and liver are microscopically found (Richman et al., 1999; Richman et al., 2000; Ehlers et al., 2001; Reid et al., 2006; Garner et al., 2009; Zachariah et al., 2013). Mortality rates of the disease have been reported of at least eighty percent. Moreover, severity of the disease depends on viral strains and the breed of the elephant. Approximately sixty percent of all deaths of Asian elephants over the past twenty years have been caused by EEHV (Hayward, 2012).

The genome of the virus is approximately 180 kb containing 116 predicted protein coding genes (Wilkie et al., 2013). At least seven strains of EEHV have been reported. The first, as EEHV1, was isolated from a fatal Asian elephant and was also found from the skin lesion of an asymptomatic case in an African elephant. Nowadays, EEHV1 has been classified into two strains, EEHV1A and EEHV1B, based on terminase gene sequences (Fickel et al., 2001). EEHV2 was isolated from an African elephant (Richman et al., 1999). EEHV3 and EEHV4 were detected from captive Asian elephants (Garner et al., 2009). EEHV5 and EEHV6 were detected from the blood of a sixty-nine-year-old female Asian elephant and a juvenile of an African elephant, respectively (Latimer et al., 2011). The seventh (EEHV7) strain was isolated from an African elephant and is available in the GenBank database (accession number JQ300082) (Hayward, 2012).

In Southeast Asia, the first case described as EEHV1 was confirmed by PCR from a three-year-old wild elephant in Cambodia in 2004 (Reid et al., 2006). Recently, EEHV1A was detected in a 2.5-year-old domestic male Asian elephant in Laos (Bouchard et al., 2014). In Thailand, thirty-one elephants were surveyed to have negative results using the PCR technique in 2005 (Hildebrandt et al., 2005). However, EEHV1 that was isolated from Thailand was confirmed by the PCR technique in 2007 and its nucleotide sequence was submitted to the GenBank database (accession number FJ515310). EEHV1A and EEHV4 were additionally revealed from a dead case and asymptomatic cases (Sariya et al., 2012; Sripiboon et al., 2013). At present, PCR combined with nucleotide sequencing has been available for routine diagnosis and strain identification (Richman et al., 2000; Fickel et al., 2001; Garner et al., 2009; Latimer et al., 2011; Atkins et al., 2013). Recently,

real-time PCR has developed as an alternative technique for strain identification (Stanton et al., 2010; Hardman et al., 2012; Sariya et al., 2012; Stanton et al., 2012). The aim of the study was to characterize the partial genome of EEHV detected from captive Asian elephants in Thailand from 2007 to 2013.

Materials and Methods

Samples and DNA Extraction: Two EDTA blood samples were collected from two captive Asian elephants (EEHV03KU11 and EEHV01KU13) and six tissue samples including tongue, lung and heart were collected from necropsies of captive Asian elephants. All samples were submitted to Kasetsart University Veterinary Teaching Hospital Kamphaensaen (KUVTH-KPS), and Kamphaensaen Veterinary Diagnostic Unit (KVDU) during 2007 to 2013 along with data of habitat, age and sex which are shown in Table 1. DNA from the samples was extracted by FavorPrep™ Viral Nucleic Acid Extraction Kit I (Favorgen®, Taiwan) based on the manufacturer's manual. The DNA samples were preserved at -20°C until used.

PCR Techniques: The strains of EEHV were identified by conventional PCR techniques using strain specific primers from previous reports including a terminase encoding gene primer of EEHV1 and EEHV2 (Richman et al., 2000). Terminase encoding genes of EEHV3 and EEHV4 were amplified (EEHV3/4 strain specific primers) by a nested PCR technique (Garner et al., 2009) where both strains were classified by nucleotide sequencing. Nested PCR techniques using specific primers with EEHV5 and EEHV6 based on DNA polymerase gene were performed (Latimer et al., 2011). Amplification by PCR techniques was performed by DreamTaq® (ThermoScientific, USA), which condition was performed according to previous reports. PCR products were separated by 1.5% agarose gel electrophoresis, stained with GelRed™ Nucleic Acid Gel stain (Biotium, USA) and visualized under UV light.

Nucleotide Sequence and Phylogenetic Tree

Construction: The PCR products were extracted from agarose gel by using NucleoSpin®ExtractII (MACHERE Y-NAGEL, Germany) according to the manufacturer's instructions and then nucleotide sequencing was analyzed at 1st Base Pte Ltd (Malaysia). The DNA sequences were determined in both directions. The nucleotide sequences of samples were confirmed by using BLAST program (<http://www.ncbi.nlm.nih.gov>). A relationship between the nucleotide sequences from this study and the GenBank database was analyzed by multiple alignment using clustalw program incorporated in the MEGA5 program and a phylogenetic tree was generated by using neighbour-joining with bootstrap 1000 from the MEGA5 program (Tamura et al., 2011). The nucleotide sequences of EEHV isolated from this study were submitted to the Genbank database under the accession numbers shown in Table 1.

Table 1 Isolates, host, age, sex, date of sample collection, country and accession number of samples from this study

Strain	Isolates	Host	Age	Sex	Date of collection	country	Accession number
1A	EEHV01KU07	<i>E. maximus</i>	1y 7 mo	F	2007	North-Eastern /Thailand	KJ607875
1A	EEHV01KU08	<i>E. maximus</i>	2y	M	2008	Southern/Thailand	FJ515310
1A	EEHV01KU11	<i>E. maximus</i>	ND	NA	2011	ND	KJ607876
1A	EEHV03KU11	<i>E. maximus</i>	ND	M	2011	Southern/Thailand	KJ607877
1A	EEHV01KU13	<i>E. maximus</i>	3y	M	2013	Southern/Thailand	KJ607878
1B	EEHV03KU09	<i>E. maximus</i>	2y	F	2009	Southern/Thailand	KJ607879
4	EEHV02KU08	<i>E. maximus</i>	1y 6mo	F	2008	Central/Thailand	KJ607880
4	EEHV03KU08	<i>E. maximus</i>	2y	F	2008	Central/Thailand	KJ607881

ND = No data

Table 2 Percentage of nucleotide identity based on partial terminase gene in strain and between strains of EEHV including nucleotide sequencing from this study and the GenBank database

Strain	EEHV1A (n=20)	EEHV1B (n=9)	EEHV2 (n=1)	EEHV3 (n=6)	EEHV4 (n=4)	EEHV5 (n=1)	EEHV6 (n=2)
EEHV1A	99-100						
EEHV1B	95.7-96.8	99.5-100					
EEHV2	70.7-71.5	72.2-72.3	100				
EEHV3	73.1-73.9	70.7-70.8	78.8	100			
EEHV4	72.5-73.3	72.5-72.6	77.4	96.3	100		
EEHV5	73.8-74.7	75.4-76.1	83.0	70.6	73.1	100	
EEHV6	85.6-86.9	86.3-86.9	68.2	67.0	70.7	73.1	100

Results

Clinical signs of all elephants were edema of the head and trunk and cyanosis of the tongue followed by death of five elephants in one week. Only two elephants survived after extensive treatment. Necropsy findings of the fatal cases included pericardial effusion and petechial hemorrhages in heart and liver, hepatomegaly, cyanosis of the tongue and intestinal hemorrhage and ulceration. Each DNA sample was amplified by using PCR techniques with strain specific primers based on terminase or DNA polymerase genes. Six of the eight samples had positive bands of the EEHV1 strain specific primers and two samples were positive for the EEHV3/EEHV4 strains. Based on the specific primers used, all DNA samples were negative for EEHV2, EEHV5 and EEHV6. Five out of the six samples of EEHV1 were positive and were classified into the EEHV1A strain and only one sample was EEHV1B (EEHV03KU09) by nucleotide sequencing and BLAST program analysis. The nucleotide sequences of two positive samples with EEHV3/4 strain specific primer were identical to that of the EEHV4 in the GenBank database. Thus, the causative EEHV of captive Asian elephants in Thailand includes three strains; EEHV1A, EEHV1B and EEHV4.

Nucleotide sequences of the viruses in this study were estimated to have a relationship with viruses isolated from other geographic areas that were submitted to the GenBank database by generation of the phylogenetic tree. The phylogenetic tree based on the terminase gene of EEHV showed a relationship among strains of EEHV between nucleotide sequences from this study and the GenBank database (Fig 1). Five EEHV1A terminase genes (193bp) containing EEHV01KU07, EEHV01KU08, EEHV01KU11, EEHV03KU11 and EEHV01KU13 from this study had identical sequences (100%) and also showed high similarity (99-100%) with EEHV1A obtained from the

GenBank database, which includes Thai isolates from previous reports; accession numbers JF742644 and FJ767711 (Sariya et al., 2012; Sripiboon et al., 2013). The EEHV1B case detected in this study is the first report in Thailand and has an identical sequence with NAP14 (accession number JN983088) isolated from an Asian elephant in Germany in 1998 and 99.5% similarity with other geographic areas. Nevertheless, the amino acid residue of the partial terminase gene of both EEHV1A and EEHV1B had identical amino acid residues.

Based on the terminase encoding gene, the nucleotides of the two isolates of EEHV4 (EEHV02KU08 and EEHV03KU08) obtained from elephants roaming in central Thailand were identical to those found in the USA (Garner et al., 2009) and Thailand (Sripiboon et al., 2013) as earlier reported. The percentage of nucleotide difference in strain and between strains is shown in Table 2. EEHV1A (five out of eight samples) had a majority of EEHV strains as the cause of EEHV infection in captive Asian elephants in Thailand.

Discussion

Captive Asian elephants have been found in all parts of Thailand and play important roles in Thai culture and the tourist industry. The number of captive Asian elephants in Thailand was 4,016 in 2010 reported by the Livestock Development Department (Mahasawangkul, 2011). Disease is one of several reasons that have had an adverse impact on elephant populations. The EEHV infection is significant because it has caused sudden deaths of young elephants in several countries (Richman et al., 1999; Richman et al., 2000; Fickel et al., 2001; Reid et al., 2006; Garner et al., 2009; Sariya et al., 2012; Atkins et al., 2013; Sripiboon et al., 2013; Zachariah et al., 2013; Bouchard et al., 2014). Young elephants (1-4 years old) had the virus detected from blood DNA that was lethal at least eighty percent

of the time (Hayward, 2012). Moreover, recent knowledge concerning pathogenesis and factors of infection have been considerably limited. EEHV has been confirmed in the deaths of elephants since 1995 and recent data have revealed that there were more than eighty cases from several different countries (Richman et al., 1999; Hayward, 2012). The virus is divided into seven strains (EEHV1-EEHV7) of which EEHV1 can also be divided into EEHV1A and EEHV1B based on nucleotide similarity. In this study, three strains including EEHV1A, EEHV1B and EEHV4 were investigated from captive Asian elephants from 2007 to 2013 using PCR techniques combined with nucleotide sequencing using specific primers based on terminase and DNA polymerase encoding genes. Both genes have been used as main targets for developing molecular techniques because the nucleotide difference among EEHV strains could be used for EEHV detection and strain identification. Richman et al. (1999) showed eighty and sixty-five percent of DNA homology of terminase and DNA polymerase gene, respectively, between EEHV1 and EEHV2. Moreover,

the percentage of nucleotide identity of terminase gene among EEHV strains in this study was 67-96.9 percent (Table 2). EEHV1A is the main causative agent in captive Asian elephants in the northeastern and southern parts of Thailand. In Thailand, Sripiboon et al. (2013) reported EEHV1A detected in the frozen heart tissue of a two-year-old male that died in 2005 (accession number JN836374). Moreover, a case of EEHV1A that was detected in a two-year-old male from Surin province in 2008 (accession number FJ767711) was deposited in the GenBank database. In 2011, EEHV1A was isolated from the heart tissue of a female Asian elephant (isolate VSMU1, accession number JF742644) and was also detected in 2 out of 29 (6.9%) from trunk swab samples of asymptomatic Asian elephants (Sariya et al., 2012). Thus, EEHV1 has a high prevalence in captive Asian elephants and can be detected in both infectious and asymptomatic elephants. Latent infection may be a high risk during an EEHV outbreak, especially under stressful and immunosuppressive conditions.

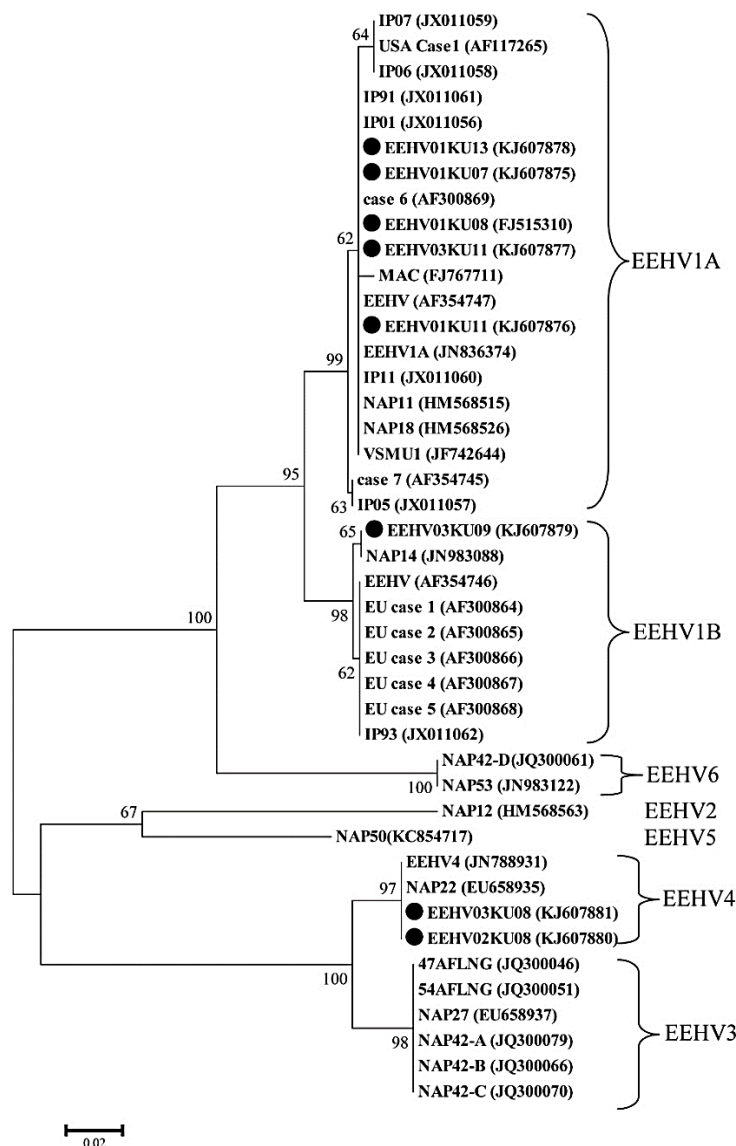


Figure 1 Phylogenetic tree based on terminase gene of DNA EEHV isolated from Asian elephants in this study (black circle). Other sequences were obtained from GenBank. Accession numbers are shown in parentheses. Bootstrap values obtained from 1000 replications are shown at branch. The scale bar represents number of substitutions for a unit branch length.

In this study, EEHV1B (EEHV03KU09) was the first strain found in Thailand which was detected from captive Asian elephants in the southern part of Thailand. EEHV1B has been found in Europe (Fickel et al., 2001) and India (Zachariah et al., 2013). One case of EEHV1B was detected in a twelve-year-old male Asian elephant born in Malaysia but was relocated to the Netherlands (Fickel et al., 2001). The EEHV4 strain was isolated from two captive elephants from central Thailand. Both viruses from this study have identical sequences to the virus (accession number JN788931) isolated from a three-year-old male Asian elephant in 2011 (Sripiboon et al., 2013). In this study, EEHV2, EEHV3, EEHV5 and EEHV6 were not positively detected. However, EEHV3 and EEHV5 were reported from Asian elephants roaming in other countries (Garner et al., 2009; Latimer et al., 2011; Atkins et al., 2013). Previous reports (Sariya et al., 2012; Sripiboon et al., 2013) and this study showed that at least ten Asian elephants died from EEHV from 2005 to 2013 by PCR confirmation of the infection. In this study, only two Asian elephants survived from the viral infection after treatment.

In conclusion, it is important to realize the role of EEHV in the high mortality rate of young elephants. Our information might lead to the improvement in herd health management, in particular, for young elephants. However, herd or captive management must also be properly practiced. Participants in elephant conservation network will play an important role in initiating consideration of EEHV control.

Acknowledgements

This work was supported by the Thailand Research Fund and Kasetsart University Research and Development Institute (TRF-MRG5480217). We would like to thank the Faculty of Veterinary Medicine, Kasetsart University for providing their facilities. We also acknowledge Sakuna Phattanakunanan, Siriluk jala and Apisit Seubsai, for sample collection and preparation

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บทคัดย่อ

ความหลากหลายทางพันธุกรรมของเชื้อเฮอร์ปีส์ไวรัสจากช้างเอเชียที่เลี้ยงในประเทศไทย ระหว่าง พ.ศ. 2550-2556

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การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาความหลากหลายของเชื้อเฮอร์ปีส์ไวรัสที่ตรวจพบในช้างเอเชียที่เลี้ยงในประเทศไทยช่วง พ.ศ. 2550-2556 จากตัวอย่างเนื้อเยื่อจากช้างเอเชียที่ล้มจำนวน 6 เชือกและตัวอย่างเลือดที่เก็บในสารป้องกันการแข็งตัวของ EDTA ของช้างเอเชียที่มีชีวิตจำนวน 2 เชือก ช้างทั้งหมดแสดงอาการทางคลินิก หรือมีรอยโรคจากการติดเชื้อไวรัสเฮอร์ปีส์ จากการตรวจสอบดีเอ็นเอที่สกัดจากตัวอย่างทั้งหมดด้วยเทคนิคพีซีอาร์โดยใช้ไพรเมอร์ที่จำเพาะกับสายพันธุ์ต่างๆของเชื้อไวรัสในส่วนของยีนเทอร์มิเนส และดีเอ็นเอพอลิเมอเรส พบว่า 6 ตัวอย่างให้ผลบวกกับไพรเมอร์สายพันธุ์ที่ 1 ส่วน 2 ตัวอย่างให้ผลบวกกับไพรเมอร์ที่จำเพาะกับทั้งสายพันธุ์ 3 และ 4 หลังจากยืนยันผลด้วยการวิเคราะห์ลำดับเบส พบว่า 5 ตัวอย่างจาก 6 ตัวอย่างที่ให้ผลบวกด้วยไพรเมอร์สายพันธุ์ที่ 1 เป็นเชื้อสายพันธุ์ EEHV1A และ 1 ตัวอย่างเป็นเชื้อสายพันธุ์ EEHV1B ส่วน 2 ตัวอย่างที่ให้ผลบวกจากไพรเมอร์ที่จำเพาะต่อทั้งสายพันธุ์ 3 และ 4 จากการวิเคราะห์ลำดับเบสของยีนเทอร์มิเนส พบว่าทั้ง 2 ตัวอย่างเป็นเชื้อสายพันธุ์ EEHV4 การศึกษาครั้งนี้พบเชื้อเฮอร์ปีส์ไวรัส 3 สายพันธุ์จากช้างเอเชียที่เลี้ยงในประเทศไทยระหว่าง พ.ศ. 2550-2556 ได้แก่ EEHV1A, EEHV1B และ EEHV4 และเป็นการศึกษาแรกที่พบเชื้อ EEHV1B จากช้างเอเชียในประเทศไทย

คำสำคัญ: ช้างเอเชีย, เฮอร์ปีส์ไวรัส, ยีนเทอร์มิเนส, ประเทศไทย

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