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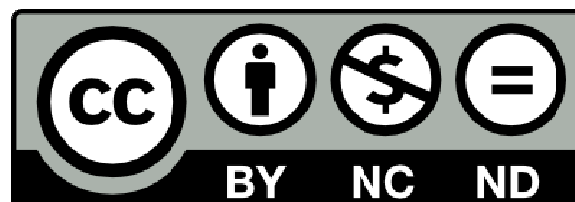

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Molecular characterisation and genetic variation of Elephant Endotheliotropic Herpesvirus infection in captive young Asian elephants in Thailand

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Abstract

Elephant Endotheliotropic Herpesvirus (EEHV) is emerging as a new threat for elephant conservation, since being identified as the cause of severe, often fatal, haemorrhagic disease in young Asian elephants. To describe positive cases and the molecular relatedness of virus detected in elephants in Thailand, we re-examined all available of EEHV samples occurring in young elephants in Thailand between 2006 and 2014 (n = 24). Results indicated 75% (18/24) of suspected cases were positive for EEHV by semi-nested PCR. Further gene analysis identified these positive cases as EEHV1A (72%, 13/18 cases), EEHV1B (11%, 2/18) and EEHV4 (17%, 3/18). This study is the first to phylogenetically analyse and provide an overview of most of the known EEHV cases that have

occurred in Thailand. Positive individuals ranged in age from one to nine years, with no sex association detected, and occurred across geographical locations throughout the country. All individuals, except one, were captive-born. No history of direct contact among the cases was recorded, and this together with the fact that various subtype clusters of virus were found, implied that none of the positive cases were epidemiologically related. These results concur with the hypothesis that EEHV1 is likely to be an ancient endogenous pathogen in Asian elephants. It is recommended that active surveillance and routine monitoring for EEHV should be undertaken in all elephant range countries, to gain a better understanding of the epidemiology, transmission and prevention of this disease.

Keywords: EEHV; Elephant; *Elephas maximus*; Herpesvirus; Thailand

Introduction

In 1999, Elephant Endotheliotropic Herpesvirus (EEHV), a new betaherpesvirus in the family *Herpesviridae*, was identified as the causative agent of an acute haemorrhagic disease in young Asian elephants (*Elephas maximus*) and African elephants (*Loxodonta* sp.) (Richman et al., 1999). EEHV has become a new and important threat to elephant conservation (Richman *et al*., 1999; Hayward, 2012) with over 60 confirmed cases in North America and Europe in the last 20 years, of which over 80% have proved fatal (Latimer *et al*., 2011; Hayward, 2012).

The disease caused by EEHV occurs in both Asian and African elephants, but occurs most frequently in juvenile Asian elephants, with an age range of affected individuals from 4 months to 18 years (Zong et al., 2007). Affected animals initially show non-specific clinical signs, such as lethargy and anorexia. Edema of the head and petechial haemorrhage at the tip of the tongue usually develop as the disease progresses, and these clinical signs are observed in most cases (Richman et al., 2000b). Animals often die within 12–72 h after the presence of clinical signs, due to the tropism of virus for endothelial cells causing vessel damage which leads to severe internal haemorrhage (Richman et al., 2000a). Death in these individuals is associated with organ failure or hypovolemic shock. Post-

mortem examination findings consistently demonstrate extensive petechial to ecchymotic haemorrhage throughout the heart and most internal organs, with intra-nuclear inclusion bodies and herpesvirus-like particles visible under light and electron microscopy, respectively (Richman *et al*., 2000a ; Richman *et al*., 2000b). The rapid progression of disease usually precludes effective treatment with only ten survivors reported to date (Hayward, 2012).

The virus has not been able to be cultured in any cell lines (Richman *et al*., 1999; Latimer *et al*., 2011); therefore, molecular-based tests are primarily used for disease diagnosis. Eight types of EEHV have been identified, with differences in pathogenicity and host preference (Hayward, 2012). Among these, the most common type is EEHV1, which has two closely related subgroups; EEHV1A and EEHV1B (Richman and Hayward, 2011). EEHV1, EEHV3, EEHV4 and EEHV5 have been reported as the causative agent for mortalities of Asian elephants (Richman *et al*., 1999; Garner *et al*., 2009; Latimer *et al*., 2011; Wilkie *et al*., 2014) whereas EEHV2, EEHV3, EEHV6 and EEHV7 are mainly found in African elephants (Richman *et al*., 1999; Latimer *et al*., 2011; Zong *et al*., 2015). Mixed infections with different types of herpesvirus have been occasionally found and reported (Latimer *et al*., 2011; Seilern-Moy *et al*., 2015; Zong *et al*., 2015).

Mortalities associated with EEHV infection were first reported in Western zoos (Ossent *et al*., 1990; Richman *et al*., 1999). Initially, an identical virus to that recovered from dead Asian elephants was also detected in the skin nodules of healthy African elephants (Richman *et al*., 1999; Richman *et al*., 2000b; Richman and Hayward, 2011). This suggested the possibility of cross-species viral transmission (Richman *et al*., 1999; Zong *et al*., 2007), and this hypothesis was also supported by the fact that the disease was first detected in Western countries at locations where Asian and African elephants were housed together (Richman *et al*., 2000b ; Ryan and Thompson, 2001). However, given the increasing number of EEHV cases reported in Asian elephants without a history of contact with African elephants, this hypothesis became more doubtful (Richman and Hayward, 2011; Zachariah *et al*., 2013).

Despite the fact that EEHV has been intensively studied in captive elephants of North America and Europe, the status of the disease in Southeast Asia, the Asian elephant's range, is not well described.

In 2006, the first confirmed case of EEHV in Southeast Asia was reported (Reid et al., 2006). The affected animal was a three year old female wild-born elephant in Cambodia, which died without any observed symptoms or treatment, however molecular testing of post-mortem samples suggested EEHV1 infection (Reid et al., 2006). Although preliminary surveillance of healthy Asian elephants in Thailand detected no herpesvirus (Hildebrandt et al., 2005), EEHV1 and EEHV4 were subsequently reported in Thailand in 2013 (Sripiboon et al., 2013). During this period, Zachariah (Zachariah et al., 2013) conducted a study of EEHV in southern India; with 9/15 samples from dead young wild or orphan Asian elephants testing positive for EEHV1, with associated pathology. No genetic relatedness was found except between two cases in elephants which lived in the same herd. This study suggested that EEHV1 was most likely to belong to a group of endogenous viruses that co-evolved with Asian elephants, rather than being transmitted from African elephants (Zachariah et al., 2013). This theory was also supported by the fact that EEHV1 was found to be shed occasionally from adult Asian elephants which showed no clinical signs (Hardman *et al*., 2012; Stanton *et al*., 2013), thus adult elephants could potentially play a role as a viral reservoir.

To obtain a better understanding of EEHV status in Asian elephant range countries, this molecular study tested all suspected EEHV cases in Thailand, to determine viral genetic diversity and investigate the epidemiological relatedness of types, subtypes and subtype clusters. The genetic characterisation of EEHV in Thai elephants was compared to cases in North America, Europe and India, which assisted in the clarification of the status of EEHV globally, and the development of an EEHV prevention plan for Asian elephants in their range countries.

Materials and methods

Samples

A retrospective molecular study was conducted to re-examine samples of suspected EEHV cases that were submitted to the four EEHV diagnostic facilities in Thailand between 2006 and 2014. These facilities included the Faculty of Veterinary Medicine, Kasetsart University; the Faculty of Veterinary

Medicine, Chiang Mai University; the Faculty of Veterinary Science, Mahidol University; and the Veterinary Research and Development Centre (lower north-eastern region).

Samples that were tested in this study included blood and tissue from 24 young Asian elephants that died unexpectedly or showed clinical signs associated with EEHV infection. General information including name, ID number, age, sex and geographical location were recorded. The individual's medical, diagnostic history and post-mortem findings were also noted. Whole blood samples had been collected from elephants while still alive or immediately after the elephants died. Necropsy tissue samples had been collected from various tissue and organs, the majority from heart and tongue. Samples were directly stored on ice or at 4 °C during transportation, and were kept in a freezer (− 20 °C/− 80 °C) until further processing.

Molecular analyses

Twenty-five grams of each tissue sample was used for DNA extraction, using a Gentra Puregene® Tissue Kit (Qiagen, Germany) following the manufacturer's protocol. A QIAamp® Blood Mini Kit (Qiagen, Germany) was used to extract the DNA from 200 µl of each whole blood sample.

All DNA samples were initially screened by first or second round semi-nested PCR using both redundant PANPOL primers and EEHV1-specific Polymerase (POL1) primers (Stanton *et al*., 2010; Latimer *et al*., 2011). Samples that gave a PCR product of the expected size with both primer pairs, as determined by gel electrophoresis, were classified as EEHV1 positive. DNA samples that did not produce a PCR amplicon with the POL1-specific primers, but were positive by PCR when using the PANPOL primers (either first or second round), were then subjected to amplification using EEHV3/4-specific primers, designed to amplify Terminase (TER3/4) and Polymerase (POL3/4) gene loci (Garner *et al*., 2009; Latimer *et al*., 2011). Samples that failed to produce an amplicon when subjected to PCR using both PANPOL and POL1 primers were considered negative for EEHV.

DNA samples that were classified as EEHV1 positive during the first step, were then amplified by PCR using four further EEHV1-specific primer pairs to obtain detailed sequence information. These four primer pairs were designed to amplify EEHV1-specific loci of the Terminase (TER1/U60),

Helicase (HEL1/U77), Glycoprotein M (gM1/U72) and G-coupled viral protein (vGPCR1/U51) genes. The primers and amplification protocols followed were as previously described (Richman *et al*., 1999; Stanton *et al*., 2010; Latimer *et al*., 2011). DreamTaq™ Green PCR master mix (Thermo Fisher, USA) was used in each PCR reaction according to the manufacturing protocol. Sterile water was used instead of DNA template as a negative control to check for any contamination.

All PCR products of the correct size were purified for sequencing using Wizard® SV Gel and PCR Clean-Up system (Promega, USA). DNA sequencing was carried out using a BigDye® Cycle Sequencing Kit (Applied Biosystems, USA) and an ABI PRISM 3130 Automated DNA Sequencer (Applied Biosystems, USA). Sequences were analysed using BioEdit® (Ibis Biosciences, USA) and compared to sequences in the database using a blastn search, to verify the anticipated EEHV identity. One reference sequence for each locus, obtained from the index case of each known EEHV type (1–6) was obtained from NCBI, pooled with sequences found in this study, and aligned with MUSCLE in MEGA6 (Tamura et al., 2013) using standard settings, and trimmed. Neighbour joining (NJ) and maximum likelihood (ML) phylogenetic trees were constructed to ascertain the type of virus in each elephant. Prior to generation of the ML tree for each locus, a best-fit substitution model was selected based on MEGA recommendation. The Tamura 3-parameter model (T92 + G) was used for gM and vGPCR loci, while the Kimura 2-parameter model (K2 + I) was used for the other loci. A thousand bootstrap replicates were performed for each tree.

The gM and vGPCR loci were more variable than the others, and this sequence variation can be used to classify EEHV1 into three subtype clusters (A, B, C for the gM locus) and five subtype clusters (A–E for vGPCR locus). One reference sequence, obtained from the virus isolated from the index case of each subtype cluster, was downloaded from NCBI and aligned with the sample sequences to generate the phylogenetic trees as described above. For the most variable locus (vGPCR), a neighbour-net, a distance method for constructing a phylogenetic network, was produced using the program SplitsTree4 (Huson and Bryant, 2006). Nucleotide substitution of Kimura 2 parameter (K2P) model was used in the analysis and branch support was estimated by bootstrapping with 1000 replicates.

A pairwise distance analysis was used to estimate the evolutionary divergence between sequences of EEHV1 positive cases, and conducted using the Jukes-Cantor model to obtain the number of base substitutions per site between sequences of each of the five EEHV1-specific loci.

Results

Between 2006 and 2014, a total of 24 cases were submitted for EEHV diagnosis. Of these, 22 were elephants that died unexpectedly, and two were clinically ill elephants. The cases were distributed across the country, and all elephants were classified as young elephants, aged < 15 years (Arivazhagan and Sukumar, 2008). Most individuals were captive-born, and of the two exceptions, one was hand-raised in captivity from six months of age.

During molecular screening, 75% of samples (18/24) were positive by PCR using the PANPOL primer pair, by either first or second round PCR. All of the EEHV positive individuals were older than one year, with the oldest being nine years of age. No significant association was found when comparing males with females (OR = 0.33, 95% CI = 0.01–9.26, $p = 0.33$), neither were juvenile elephants (< 5 years old) more likely to be positive by PCR than elephants > 5 years old (OR = 5.33, 95% CI = 0.26–110.8, $p = 0.24$). Positive cases were distributed across Thailand (Fig. 1), and all cases were from different captive elephant facilities. The details of each positive case are listed in Table 1.

Among the group that was PCR positive, 16 of the samples were from elephants that had died during the acute phase of the disease, and two were from clinically ill elephants. Clinical signs associated with EEHV infection, including facial swelling, tongue cyanosis and petechial haemorrhage at the tip of the tongue, were observed in the majority of elephants that died, as well as the two individuals that had suffered from clinical illness associated with the infection. Lameness was observed in one case (ThE09) and bloody diarrhoea was also presented in one case (ThE18). Only 12 cases were available for post-mortem examination, and the necropsy examination of these individuals revealed moderate to extensive haemorrhage in the heart and most of the internal organs.

Six of the 24 cases screened were mortality cases that had previously tested negative for EEHV. These included a baby wild elephant found dead in the forest; a newborn baby that had been attacked by its mother; a late-term still born elephant; and three baby elephants that died with an unclear history. None of the samples tested from these elephants were positive by molecular screening in our study.

To determine the types of EEHV in each elephant, the polymerase/U38 locus of all samples were aligned with reference sequences, using a blastn search. The alignment contained 209 parsimony informative sites out of 439 positions. For both NJ and ML phylogenetic analysis our sequences clustered with the EEHV1A, EEHV1B and EEHV4 reference sequences, creating clear clusters with high support values (Fig. 2). Genetic subtyping revealed that 72% (13/18) of positive cases were closely related to EEHV1A, 11% (2/18) to EEHV1B, and 17% (3/18) to EEHV4.

Further detailed genetic analysis for EEHV1-specific loci was conducted in order to clarify the genetic variation and relatedness between each of the isolates. Consistent genetic subtyping results were obtained over multiple loci (POL1, TER1, HEL1, gM1), where samples can be classified into EEHV1A and EEHV1B subtypes. However, the most variable locus (vGPCR) revealed sufficient genetic variation to allow identification of several previously identified subtypes in our isolates and altogether, five different subtype clusters of EEHV1 (A–E) were found in this study, with the most common subtype cluster being type D (6/15). Based on analysis of the vGPCR1 locus, NJ and ML phylogenetic tree were generated, however, low bootstrap values were observed indicating that the taxon was unreliable, thus a neighbour-net phylogenetic network was then generated to show any relatedness of the EEHV isolates found in Thailand (Fig. 3).

Analysis of the five different loci of EEHV1 (POL1, TER1, gM1, HEL1, vGPCR1), showed that none of the isolates were identical over all of these loci, indicating that there was no epidemiological relatedness between these cases. The number of base substitutions per site between sequences is shown in Fig. 4. However, we found that the EEHV4 genomes were well conserved with only a 1% difference, based on polymerase gene analysis.

Discussion and conclusion

In this study, we undertook genetic characterisation and phylogenetic analysis of the majority of EEHV cases that had occurred in Thailand during 2006–2014. This study was the first to undertake phylogenetic analysis and to provide an overview of most of the known and suspected cases of EEHV that had occurred in Thailand. A total of 18 cases were confirmed as positive for EEHV infection, with only two elephants surviving the infection after intensive treatment with human anti-viral drugs. EEHV1A was the most common viral subtype found in Thailand, occurring in 72% of all positive cases. The common detection of this subtype was also previously reported from North America, Europe and India (Richman and Hayward, 2011; Hayward, 2012; Zachariah *et al*., 2013). When the results from this study were combined with those of a previous study (Lertwatcharasarakul et al., 2015) and repeat samples were excluded, the data indicated that a minimum of 21 confirmed cases of EEHV had been recorded in Thailand.

This study revealed no association between the location and sex of elephants that were affected, which was similar to the results of previous studies (Latimer *et al*., 2011; Richman and Hayward, 2011), although we note the issue for detecting an effect with small sample sizes (O'Brien et al., 2009). The affected elephants submitted to this study were all classed as young elephants, and this age bias is a limitation for detecting any age association with disease caused by EEHV. However given that an age association has been suspected and reported elsewhere (Zong *et al*., 2007; Hayward, 2012), and that the majority of confirmed EEHV cases were juvenile elephants in this study, we recommend routine monitoring for EEHV in juvenile elephants especially, including daily observation for clinical signs, with PCR screening of any individuals suspected to be in the early stages of the disease. For these reasons, it is important to train captive elephants from a young age to undergo sampling for diagnostic and medical purposes. Furthermore, routine surveillance of the herd should be conducted to determine the viral status of individuals across all age classes. This will potentially detect any carriers or actively shedding individuals, as well as providing much needed clarification on the epidemiology of the disease in captivity.

Previous studies have shown that EEHV also occurs in wild elephant populations in India (Zachariah et al., 2013), however in our study, all of the positive cases were captive-born elephants, with one exception of a wild-born elephant raised in captivity from six months of age. However, the disease was identified in this individual during captivity, so whether the source of the virus was from the wild or a captive animal was unclear. In this study, the only truly wild individual sample was a baby elephant found dead in the forest; and the result was negative for EEHV. Given the limited sampling of wild elephants, we cannot infer the population status for wild Asian elephants in Thailand. There is a need for targeted herpesvirus surveillance of wild populations to determine their disease status, as well as a need to develop appropriate sampling techniques in remote areas, as the areas inhabited by wild elephants are often difficult to access.

Clinical signs associated with EEHV, including facial swelling, cyanosis of the tongue and petechial haemorrhages at the tip of the tongue, were observed in the majority of elephants that died of acute disease or were clinically ill. In case ThE18, which was positive for EEHV4, bloody diarrhoea was also present, supporting the observation that this type of virus shows less selective organ tropism than other subtypes (Garner *et al*., 2009; Sripiboon *et al*., 2013). Among the disease-positive group, most of the tested samples, including the blood and tongue samples, were positive when tested by first round PCR using the PANPOL primer pair. This demonstrated that the protocol was suitable for screening for EEHV in clinical cases using blood samples. We also recommend that in situations where full necropsy cannot be performed, at least tongue samples should be collected for disease diagnosis. In this study, viral DNA was successfully recovered from tissues kept in a freezer (− 80 °C) for more than five years, indicating that this was a viable system for sample storage for retrospective studies.

Detailed genetic analysis is a helpful tool for molecular epidemiological studies, and Zong et al. (2007) undertook the first EEHV genetic subtyping of 15 EEHV confirmed cases that occurred in North America. The results revealed that none of the cases were related epidemiologically, despite the fact that some elephants were kept in the same facilities as each other, or had the same parents (Zong et al., 2007). However in India, a common epidemiological source was suspected, with identical virus

sequences in two dead elephants that lived in the same facility (Zachariah et al., 2013). In our study, various subtype clusters were also found and none of the viral isolates were genetically identical over multiple loci analysis. Although none of the cases in this study were from the same herd at the time that disease occurred, some of the elephants had a history of sharing the same facilities at least once in their lifetime (ThE01, ThE09, and ThE15). However, sequence analysis of several PCR amplified loci revealed that viruses isolated from these elephants were not identical (Fig. 3). This is suggested that a single locus analysis may not be sufficient to draw a conclusion concerning epidemiological relatedness and multiple gene analysis is needed. EEHV is shed in elephant secretions including those from trunk and conjunctiva (Hardman *et al*., 2012; Atkins *et al*., 2013; Stanton *et al*., 2013), with evidence this shedding pattern is intermittent (Hardman et al., 2012), thus it is likely there is ongoing exposure within a herd. The findings from our study also suggested that disease occurrence is sporadic despite the high likelihood of all animals encountering the virus, and therefore the development of disease following EEHV infection likely depends on the host immune response.

As mentioned previously, the most commonly detected type of EEHV is EEHV1, therefore to evaluate the molecular epidemiology of EEHV1, further gene analysis is required. Five EEHV1-specific loci (TER1, POL1, HEL1, gM1 and vGPCR1) were used to obtain detailed genetic information for each isolate. Among these five EEHV1-specific loci, gM1 and vGPCR1 were more divergent when compared to the others. Previous studies showed three major subtype clusters of EEHV1 (cluster A, B, and C) can be distinguished by gM1 gene locus comparison (Zachariah et al., 2013), whereas five major subtype clusters, including A–E can by distinguished by analysis of the vGPCR1 gene sequence (Zong *et al*., 2007; Zachariah *et al*., 2013). Our study revealed only two subtype clusters of gM1 (A and B). However using the vGPCR1 gene locus, all five subtype clusters were identified, with different frequencies for each type (Fig. 3). Our results were similar to those seen in the North American cases (Zong et al., 2007), where the most common subtype clusters were cluster A and C (Table 1). However, this finding was different from that reported in southern India (Zachariah et al., 2013), where the most common subtype cluster was cluster E (in contrast, in Thailand only one case of cluster E was found). Furthermore, our study reported one case of cluster C

(THE12), while no cases of cluster C had been reported in Europe and southern India (Zachariah et al., 2013).

Only EEHV1 and EEHV4 were found in this study, which concurred with a previous report (Sripiboon et al., 2013). Surveillance for subclinical infection was recently conducted on healthy captive and semi-captive Asian elephants in southern India, where results revealed that the most common viral type to be shed from animals was EEHV5 (Stanton et al., 2014). However, the number of fatal cases associated with EEHV5 infection was relatively low, compared to its high prevalence in healthy/subclinical elephants (Latimer *et al*., 2011; Atkins *et al*., 2013; Stanton *et al*., 2014), with only one fatal case reported in association with EEHV5 infection (Denk *et al*., 2012; Wilkie *et al*., 2014). These results were similar to those from our study, where none of the fatal cases were due to EEHV5 infection. This suggests that EEHV5 could be another ancient endogenous pathogen in Asian elephants, and the low pathogenicity could be due to a long history of host-pathogen co-evolution, contributing to either better host adaptive immune response or low viral pathogenicity, and it is possible that infection with EEHV5 could possibly be protective against infection with a more pathogenic type. Future studies should focus on clarifying the prevalence of different types and subtypes of EEHV in healthy captive and wild Asian elephants in Thailand across different regions. Moreover, given the limitations of virus detection in healthy elephants based on intermittent shedding, surveillance should comprise both molecular and serological methods to improve detection probabilities and better characterise the epidemiology of this disease in the population.

Mixed infection with different types of EEHV has been recently reported in a fatal case and in healthy elephants (Seilern-Moy *et al*., 2015; Zong *et al*., 2015). Based on the unambiguous chromatograms obtained by direct sequencing the PCR products amplified by the redundant PANPOL primers in this study, no mixed infection was apparent. However, it was possible that PCR using these redundant primers tended to pick up only the dominant type of EEHV, if there was any co-infection (Zong et al., 2015). Therefore, the results from our study only refer to the dominant type that was more likely to be causing the disease in the elephant at the time of sampling. Moreover, differences in efficiency of detection of each type when using these primers have been previously reported (Latimer *et al*.,

2011; Zong *et al*., 2015). Therefore, to rule out any co-infection with other types of EEHV, type-specific primers are required and we recommend their use in the future.

Elephant herpesvirus infection is currently one of the biggest challenges for elephant conservation, as the disease has been responsible for at least 60% of all deaths in young captive Asian elephants over the past 20 years (Hayward, 2012). Asian elephants in their home ranges not only play an important role in the ecosystem; they are also involved in the life of the populace, its culture and tradition. Therefore disease threats to this species are important to characterise not only for conservation purposes, but also to mitigate the impact they may have on national and cultural icons, and traditional ways of life and employment in human communities throughout the region. Our study examined the largest number of confirmed clinical cases of EEHV infection in SE Asia, and identified the occurrence of EEHV infection in a home-range country of Asian elephants. Clarifying the genetic characterisation and epidemiology of this virus and associated disease in captivity will enable the development and implementation of appropriate risk mitigation steps, including recommended screening protocols for herd management. This study did not find any epidemiological relatedness among cases and the occurrence of disease appears to be sporadic. Furthermore, no history of contact with African elephants was recorded in this study; supporting the hypothesis that the virus is an endogenous pathogen in Asian elephants. If this is true, the recent increase in reported clinical cases associated with the virus may be related to: i) the recent availability of molecular diagnostic facilities; ii) increasing virulence of the virus; or iii) changes in factors that influence the host immune response; for example pre-disposing stressful events or concurrent infection. Capacity building, laboratory knowledge transfer, data sharing, and regional and international collaboration are also important, and need to be established in the Asian region in the near future to facilitate research on EEHV.

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Fig. 1. The distribution of 18 EEHV positive cases found in this study, each dot indicates a case.

Fig. 4. Estimates of evolutionary divergence between sequences, showing the number of base substitutions per site between sequences at each locus including (a) POL1/U38, (b) TER1/U60, (c) HEL1/U77, (d) gM1/U71, and (e) vGPCR1/U51.

(a)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
ThE01_1A-POL	-														
ThE02_1A-POL	3	-													
ThE03_1A-POL	7	7	-												
ThE04_1B-POL	44	32	38	-											
ThE05_1A-POL	0	3	3	28	-										
ThE06_1A-POL	0	2	4	34	0	-									
ThE07_1A-POL	3	7	9	38	6	4	-								
ThE08_1A-POL	3	2	9	34	6	4	9	-							
ThE09_1A-POL	0	2	4	34	0	0	4	4	-						
ThE10_1B-POL	48	34	42	2	31	36	41	36	36	-					
ThE11_1A-POL	0	2	4	34	0	0	4	4	0	36	-				
ThE12_1A-POL	0	2	4	34	0	0	4	4	0	36	0	-			
ThE13_1A-POL	0	3	5	33	0	0	3	3	0	36	0	0	-		
ThE14_1A-POL	0	2	4	34	0	0	4	4	0	36	0	0	0	-	
ThE15_1A-POL	0	2	4	34	0	0	4	4	0	36	0	0	0	0	-

(b)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
ThE01_1A-TER	-														
ThE02_1A-TER	0	-													
ThE03_1A-TER	4	4	-												
ThE04_1B-TER	33	33	38	-											
ThE05_1A-TER	0	0	4	33	-										
ThE06_1A-TER	0	0	4	33	0	-									
ThE07_1A-TER	17	17	21	38	17	17	-								
ThE08_1A-TER	0	0	4	33	0	0	17	-							
ThE09_1A-TER	0	0	4	33	0	0	17	0	-						
ThE10_1B-TER	4	4	8	38	4	4	17	4	4	-					
ThE11_1A-TER	4	4	0	38	4	4	21	4	4	8	-				
ThE12_1A-TER	0	0	4	33	0	0	17	0	0	4	4	-			
ThE13_1A-TER	0	0	4	33	0	0	17	0	0	4	4	0	-		
ThE14_1A-TER	0	0	4	33	0	0	17	0	0	4	4	0	0	-	
ThE15_1A-TER	8	8	12	33	8	8	17	8	8	12	12	8	8	8	-

(c)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
ThE01_1A-HEL	-														
ThE02_1A-HEL	3	-													
ThE03_1A-HEL	5	3	-												
ThE04_1B-HEL	25	24	26	-											
ThE05_1A-HEL	3	3	5	22	-										
ThE06_1A-HEL	5	3	5	24	3	-									
ThE07_1A-HEL	8	5	5	22	5	5	-								
ThE08_1A-HEL	4	4	4	21	0	2	2	-							
ThE09_1A-HEL	5	5	3	24	2	5	5	0	-						
ThE10_1B-HEL	25	24	26	0	22	24	22	21	24	-					
ThE11_1A-HEL	3	3	5	22	0	3	5	0	2	22	-				
ThE12_1A-HEL	3	2	3	22	2	2	3	2	3	22	2	-			
ThE13_1A-HEL	15	14	14	14	12	14	8	11	12	14	12	12	-		
ThE14_1A-HEL	15	10	10	20	10	10	5	9	10	20	10	8	7	-	
ThE15_1A-HEL	3	2	3	22	2	2	3	2	3	22	3	0	12	8	-

(d)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
ThE01_1A-gM	-														
ThE02_1A-gM	0	-													
ThE03_1A-gM	0	0	-												
ThE04_1B-gM	21	21	21	-											
ThE05_1A-gM	3	3	3	25	-										
ThE06_1A-gM	0	0	0	21	3	-									
ThE07_1A-gM	3	3	3	24	0	3	-								
ThE08_1A-gM	0	0	0	21	3	0	3	-							
ThE09_1A-gM	3	3	3	24	0	3	0	3	-						
ThE10_1B-gM	21	21	21	0	25	21	24	21	24	-					
ThE11_1A-gM	0	0	0	21	3	0	3	0	3	21	-				
ThE12_1A-gM	0	0	0	21	3	0	3	0	3	21	0	-			
ThE13_1A-gM	0	0	0	21	3	0	3	0	3	21	0	0	-		
ThE14_1A-gM	3	3	3	25	0	3	0	3	0	25	3	3	3	-	
ThE15_1A-gM	3	3	3	24	7	3	7	3	7	24	3	3	3	7	-

(d)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	cluster
ThE01_vGPCR	-															A2
ThE02_vGPCR	33	-														D2
ThE03_vGPCR	33	0	-													D2
ThE04_vGPCR	38	41	41	-												B3
ThE05_vGPCR	15	25	25	37	-											A3
ThE06_vGPCR	54	29	29	61	51	-										D1
ThE07_vGPCR	33	0	0	41	25	29	-									D2
ThE08_vGPCR	0	33	33	38	15	54	33	-								A2
ThE09_vGPCR	47	22	22	53	45	9	22	47	-							D1
ThE10_vGPCR	33	41	41	15	34	57	41	33	50	-						B1
ThE11_vGPCR	0	33	33	38	15	54	33	0	47	33	-					A2
ThE12_vGPCR	29	43	43	53	37	43	43	29	47	45	29	-				C
ThE13_vGPCR	0	33	33	38	15	54	33	0	47	33	0	29	-			A2
ThE14_vGPCR	51	26	26	59	49	3	26	51	12	54	51	43	51	-		D1
ThE15_vGPCR	37	35	35	52	34	30	35	37	26	48	37	24	37	30	-	E

Fig. 2. Based on the polymerase/U38 locus, 209 parsimony informative sites out of 439 positions were apparent in multiple alignments, using MEGA6. A phylogenetic dendrogram was constructed using the maximum likelihood method based on the Kimura 2-parameter model showing the relationship of all EEHV positive samples in Thailand (except ThE18), showing that the most common type was EEHV1A. Bootstrap values are indicated in each branch. (*) indicates reference sequences.

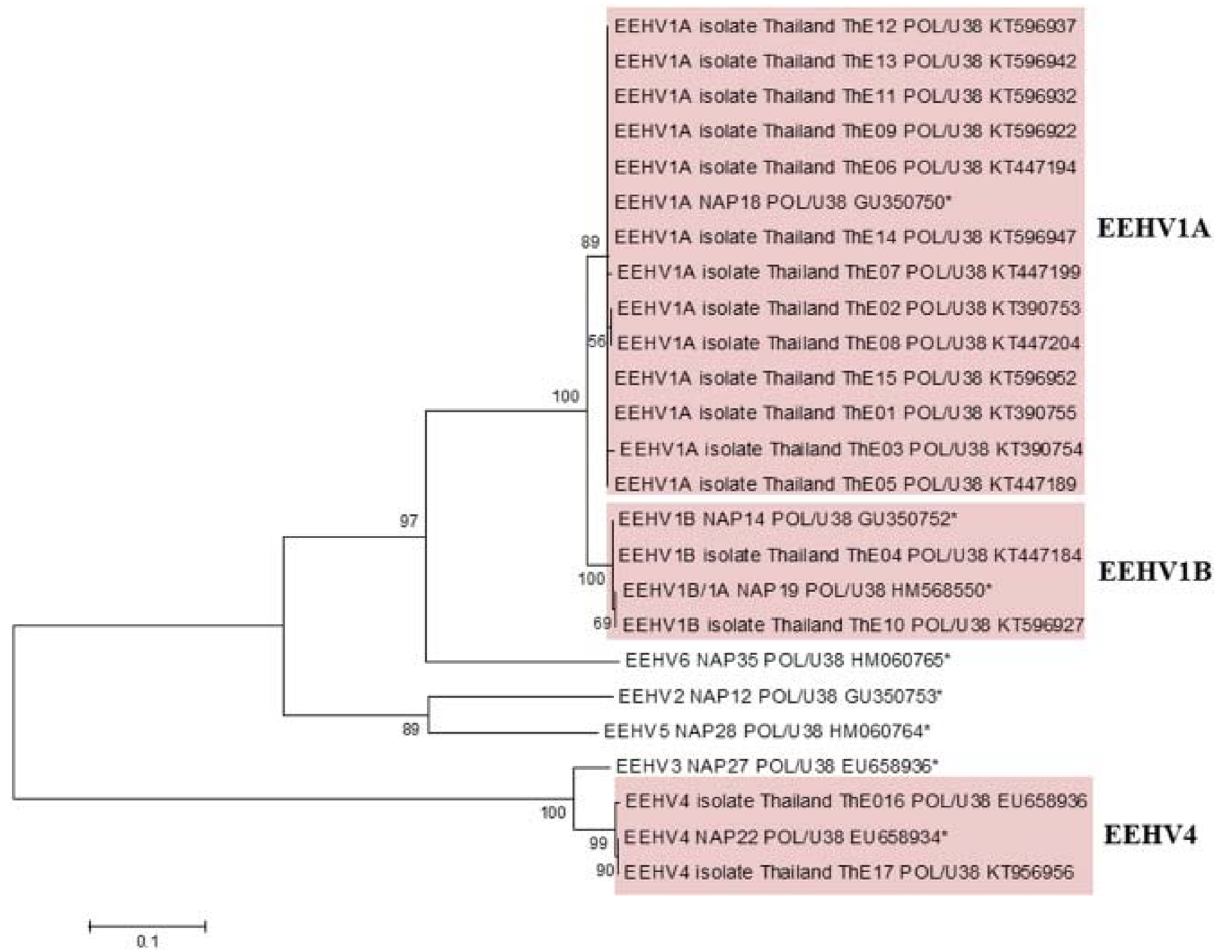

Fig. 3. Based on the vGPCR/U51 locus, 221 parsimony informative sites out of 575 positions were apparent in multiple alignments, using MEGA6. A neighbour-net was constructed (using SplitsTree4, with bootstrap values) from all EEHV1 positive cases and the reference sequences. The phylogenetic network in the square box shows the five different subtypes clusters of EEHV1 found in this study. (*) indicates reference sequences; (_) indicates elephants that had a history of sharing the same facilities at least once in their life time.

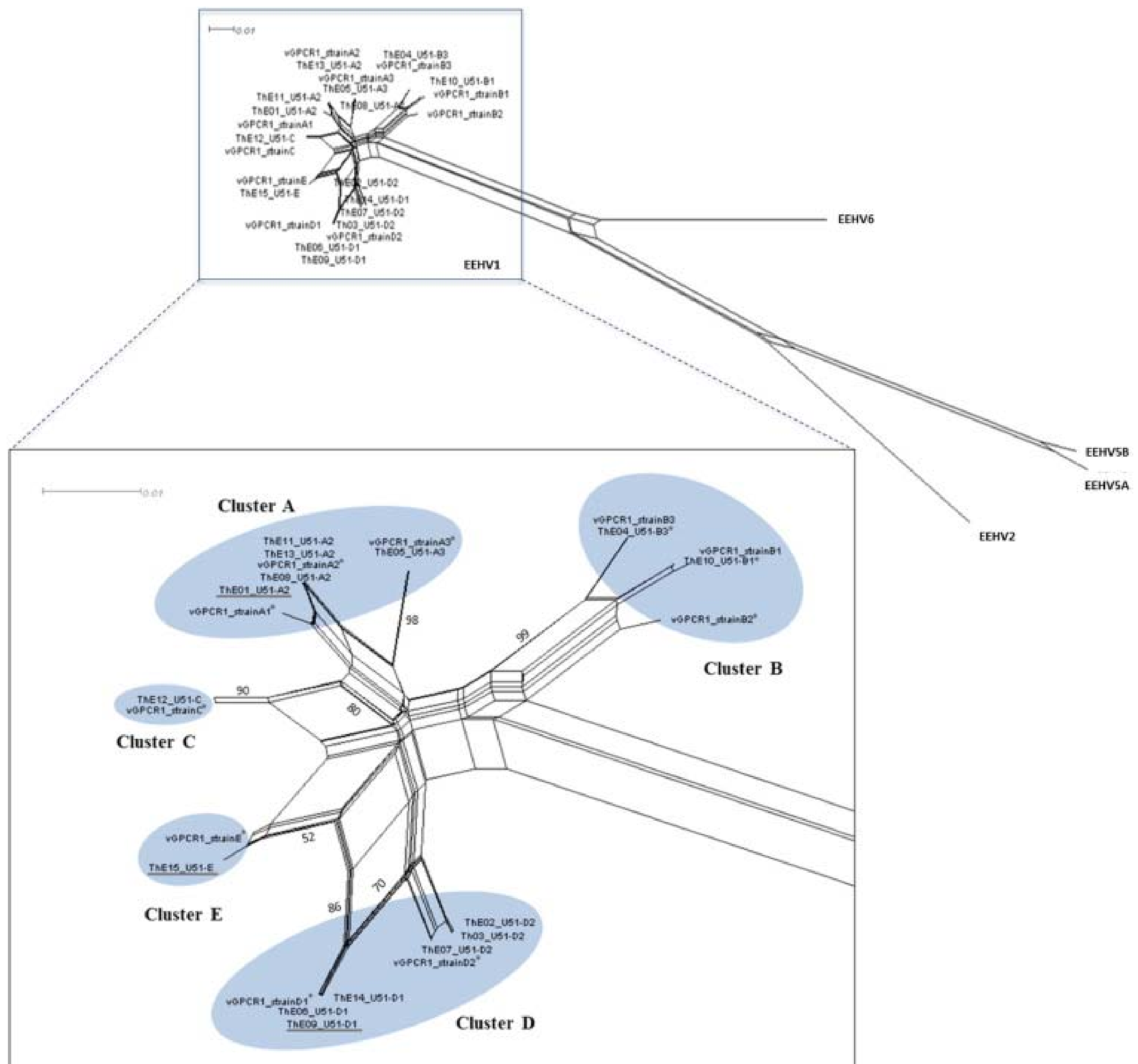

Table 1. Detail of each EEHV positive case confirmed in this study.

Isolate	YOC[a]	Type of sample	Sex	Age	Location in Thailand	Status	Outcome	Lab result	Subtype cluster[b]	Genbank accession NO.
ThE01	2006	Heart	M	2 yr	Central	Captive born	Dead	EEHV1A	A2	KT390749, KT390752, KT390755, KT390758, KT390761
ThE02	2008	Heart	M	2 yr	South	Captive born	Dead	EEHV1A	D2	KT390747, KT390751, KT390753, KT390756, KT390760
ThE03	2008	Heart	M	2 yr 2 mt	North-east	Captive born	Dead	EEHV1A	D2	KT390748, KT390750, KT390754, KT390757, KT390759
ThE04	2009	Heart	F	2 yr	South	Captive born	Dead	EEHV1B	B3	KT447182-KT447186
ThE05	2011	Heart	F	5 yr	East	Captive born	Dead	EEHV1A	A2	KT447187-KT447191
ThE06	2012	n/a	M	1 yr 2 mt	North-east	Captive born	Dead	EEHV1A	D1	KT447192-KT447196
ThE07	2012	n/a	F	2 yr 5 mt	North-east	Captive born	Dead	EEHV1A	D2	KT447197-KT447201
ThE08	2013	Tongue	n/a	n/a	North	Captive born	Dead	EEHV1A	A2	KT447202-KT447206
ThE09	2013	Heart	F	9 yr	North	Captive born	Dead	EEHV1A	D1	KT596920-KT596924
ThE10	2013	Tongue	F	1 yr 1 mt	West	Captive born	Dead	EEHV1B	B1	KT596925-KT596929
ThE11	2013	Tongue	F	1 yr 5 mt	West	Captive born	Dead	EEHV1A	A2	KT596930-KT596934
ThE12	2013	Tongue	M	2 yr 6 mt	West	Captive born	Dead	EEHV1A	C	KT596935-KT596939
ThE13	2013	Blood	M	2 yr	South	Captive born	Survived	EEHV1A	A2	KT596940-KT596944
ThE14	2014	Tongue	F	1 yr 2 mt	North	Captive born	Dead	EEHV1A	D1	KT596945-KT596949
ThE15	2014	Blood	M	3 yr	North	Wild born	Survived	EEHV1A	E	KT596950-KT596954
ThE16	2008	Heart	M	1 yr 4 mt	North-east	Captive born	Dead	EEHV4	–	KT596955
ThE17	2014	Heart	F	3 yr 8 mt	North	Captive born	Dead	EEHV4	–	KT956956, KT956957
ThE18	2011	Heart	M	2 yr 11 mt	North	Captive born	Dead	EEHV4	–	JN78893

[a] Year of sample collection

[b] Subtype cluster based on vGPCR1 (EEHV type1-specific locus).