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Research paper

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

Supaphen Sripiboon [a,d,*], Bethany Jackson [a], William Ditcham [a], Carly Holyoake [a], Ian Robertson [a], Chatchote Thitaram [b,c], Pallop Tankaew [b], Preeda Letwatcharasarakul [d], Kristin Warren [a]

[a] College of Veterinary Medicine, School of Veterinary and Life Sciences, Murdoch University, Murdoch, 6150, Western Australia, Australia
[b] Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai 50100, Thailand
[c] Center of Excellence in Elephant Research and Education, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai 50100, Thailand
[d] Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, 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.

1. 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, 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

* Corresponding author at: College of Veterinary Medicine, School of Veterinary and Life Sciences, Murdoch University, Murdoch, 6150, Western Australia, Australia.
E-mail addresses: ssripiboon@gmail.com, s.sripiboon@murdoch.edu.au (S. Sripiboon).

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(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, 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.

2. Materials and methods

2.1. 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.

2.2. 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