Elephant Endotheliotropic Herpesvirus Infection in Captive Asian Elephants (*Elephas maximus*) in Thailand: Implications for Conservation and Health Management

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A dissertation submitted to Murdoch University in fulfilment of the requirement of a Doctor of Philosophy

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November 2016
“You cannot get through a single day without having an impact on the world around you. What you do makes a difference, and you have to decide what kind of difference you want to make.”

-Jane Goodall
I declare that this thesis is my own account of my research and contains as its main content, work which has not been previously submitted for a degree at any tertiary education institution.

Dr Supaphen Sripiboon DVM MSc

November 2016
ACKNOWLEDGEMENTS:

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Last but not least, my deepest gratitude goes to my family for their never ending love and support throughout my life and my studies. I would like to particularly give thanks to my mum who started growing me with a seed of education; even though she is not here with me anymore, but this thesis is purely for her, and she is my inspiration for doing this and every good thing. Thanks to my sister, who always stands beside me and understands me even when I haven’t spoken a word.

Lastly too, thanks to all my lovely long-nosed giant friends. I am hoping that my research will help to conserve this incredible species.
ABSTRACT:

Elephant Endotheliotropic Herpesvirus (EEHV) infection is of conservation concern to endangered Asian elephants (*Elephas maximus*), causing severe, acute, often fatal haemorrhagic disease in young elephants. This thesis investigates the epidemiological status of clinical and subclinical EEHV infection in Thailand, through a retrospective, a cross-sectional, and a longitudinal study in captive elephants. Novel diagnostic tools were developed and recommendations discussed, with a particular focus on practical recommendations to manage EEHV infection in Thailand, where logistical and management constraints can hamper disease diagnosis and timely treatment.

The retrospective study revealed at least 18 EEHV clinical cases in Thailand (2006–2014), with only two cases surviving following intensive treatment. Viral samples from each case were not identical based on multiple gene analysis; suggesting the disease is likely to be sporadic. The findings also suggest that EEHV1A and EEHV1B are likely endogenous pathogens in Asian elephants.

A novel SYBR green I-based real-time PCR assay was developed to identify subclinical infection, diagnose early infection, and monitor disease progression. This technique provided sufficient data, with appropriate detection limits to differentiate at least three types of EEHV (EEHV1A, 1B, and 4). Applying this technology to the cross-sectional study, conducted between 2013–2015, revealed a 5.5% prevalence of asymptomatic EEHV1 infection during this study period in captive Asian elephants in Thailand. Cross-sectional analysis did not find an association between EEHV infection and sex, location, or contact history with other EEHV-positive elephants; however, EEHV was more likely
to be detected in juveniles than other age classes (OR = 4.46; 95%CI: 1.60–12.45; p = 0.05).

The study also monitored EEHV shedding patterns longitudinally in an EEHV-positive herd. Frequency of EEHV detection varied within and between individuals, but was significantly higher in elephants which had survived previous clinical EEHV infection (OR = 4.85; 95%CI: 0.88–26.74; p = 0.05). Concurrent monitoring of faecal corticosterone metabolites demonstrated that EEHV activated and reactivated spontaneously and shed sporadically, despite lack of obvious stress.

This thesis describes the first intensive EEHV study in an Asian elephant range country. The thesis assesses the novel real-time PCR protocol and current diagnostic tools, and recommends practical management and disease preparedness strategies to minimise the impacts of EEHV on wild and captive populations, both locally and internationally.
PUBLICATIONS:

Journal articles:


In preparation:


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<table>
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<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Adult elephant</td>
<td>older than 15 years old</td>
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<tr>
<td>Avg</td>
<td>average (mean)</td>
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<tr>
<td>Baby elephant</td>
<td>newborn to one year of age</td>
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<tr>
<td>Blastn</td>
<td>basic local alignment search tool (nucleotide)</td>
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<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>ºC</td>
<td>degrees celsius</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
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<td>cm</td>
<td>centimetre</td>
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<td>cPCR</td>
<td>conventional polymerase chain reaction</td>
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<td>Ct</td>
<td>threshold cycle</td>
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<td>CV</td>
<td>coefficient of variation</td>
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<td>deoxynucleotide triphosphates</td>
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<td>dsDNA</td>
<td>double-stranded deoxyribonucleic acid</td>
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<td>EDTA</td>
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<tr>
<td>EEHV</td>
<td>Elephant Endotheliotropic Herpesvirus</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td><em>Elephas maximus</em></td>
<td>scientific name for Asian elephant</td>
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<td>F</td>
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<td>IUCN</td>
<td>the International Union for Conservation of Nature</td>
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<td>Juvenile elephant</td>
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<tr>
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<td>no template control</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
</tbody>
</table>
ON occasionally negative
OP occasionally positive
OR odds ratio
$p$ p-value or probability value
PBS phosphate buffered saline
PCR polymerase chain reaction
POL polymerase
Pos positive
qPCR quantitative polymerase chain reaction (real-time PCR)
rpm round per minute
RN remain negative
RP remain positive
SD standard deviation
Species 360 formerly named International Species Information System or ISIS
Subadult elephant older than five years old, but younger or equal to 15 years old
TER terminase
TK thymidine kinase
$T_m$ melting temperature
$\mu g$ microgram
$\mu l$ microliter
$\mu M$ micromolar
UK United Kingdom
USA United States of America
VGCs viral genome copies
vGPCR viral G protein-coupled receptor
Young elephant older than one year, but younger than 15 years
CHAPTER 1

INTRODUCTION
1.1 Background to the study

Southeast Asia is currently experiencing a dramatic decline in wildlife species due to factors including habitat loss and modification, habitat fragmentation, illegal trading, poaching, and disease (Sodhi et al., 2004; Sinclair et al., 2006; Corlett, 2007; Koh & Sodhi, 2010). Disease, leading to alterations in health status, can pose a significant challenge to individual species and populations, especially those already compromised by other threatening agents (Scott, 1988; Thompson et al., 2010). Disease has been shown to be the cause of significant population decline in a number of endangered species, including the black-footed ferret (*Mustela nigripes*), due to canine distemper virus; the heath hen (*Tymphanuchas cupido cupido*), due to blackhead; the Indian wild ass (*Equus hemionus khur*), due to surra and African horse sickness; and more recently, the saiga antelope (*Saiga tatarica*) due to haemorrhagic septicaemia (*Pasteurella multocida*) (Caughley & Gunn, 1996; Miler-Gulland, 2015). Disease, apart from causing death, can also increase the susceptibility to predation, lower the reproductive capacity, and increase susceptibility to other diseases, or lead to a combination of these outcomes (Spielman, 2001). Disease can become catastrophically important to endangered species where population levels are critically low (Harwood & Hall, 1990). These populations can also lose genetic variability as a result of random stochastic processes (i.e. genetic drift) or inbreeding, and this may in turn increase susceptibility to disease (Hutchins et al., 1991).

Understanding the disease status of wildlife populations is an essential part of wildlife conservation. Disease monitoring and surveillance play a pivotal role for wildlife managers aiming to increase population numbers, especially when management techniques including captive breeding and translocation are employed. These techniques can facilitate the spread of disease, if appropriate precautions are not taken. Studies are
needed to assess the health and disease risks to the target species in its environment and free-ranging co-habitants.

The Asian elephant (*Elephas maximus*) is listed as an ‘Endangered’ species (IUCN, 2008). The main threat to the Asian elephant is habitat loss, degradation, and fragmentation associated with the expansion of urban areas and human populations (Choudhury et al., 2008); with the latter invariably leading to increased human-elephant conflicts. Disease is an additional threat leading to elephant population declines, either through direct morbidity or mortality, or through indirect impacts on immune function and body fitness (Hedges, 2006; Mikota, 2006a; Mikota & Fowler, 2006).

Elephant Endotheliotropic Herpesvirus (EEHV) is a recently recognised subgroup of herpesviruses, which can cause severe acute haemorrhagic disease (EEHV HD), often fatal in young Asian elephants (Hayward, 2012; Long et al., 2015). In the past 20 years, this disease has been responsible for up to 60% of fatalities in captive young Asian elephants in North America and Europe (Hayward, 2012). As a consequence, in the absence of effective management, this disease could potentially drive population decline and species extinction in the near future (Richman et al., 1999; Hayward, 2012; Long et al., 2015). The disease has been intensively studied in Western countries; however, despite recent reports of EEHV HD in both wild and captive elephant populations in many countries in Southeast Asia, including Thailand, India, Lao People’s Democratic Republic (PDR), Myanmar, Indonesia, and Cambodia, its status in these countries is not well described (Long et al., 2015).

EEHV HD has a rapid progression, with death often occurring within 12–72 hours of the onset of clinical signs (van den Doel et al., 2015). Treatment of the disease is challenging, involving antiviral drugs usually used in human medicine with low and unpredictable
success rate (Wiedner et al., 2012). Only ten surviving elephants had been reported from the late 1990’s to 2012 (Hayward, 2012). Diagnostics for EEHV infection are molecular-based, as attempts to grow the virus in cell lines have proven unsuccessful (Latimer et al., 2011; Hayward, 2012; Richman & Hayward, 2012). Development of a vaccine is still undergoing further research (Bennett et al., 2015b). Therefore at present, the prevention of EEHV HD relies largely on intensive disease monitoring.

Understanding status of EEHV HD and risk factors associated with a clinical infection will enable the improvement of disease monitoring and preventative management plans for Asian elephants. This thesis reports the first intensive study on EEHV infection in Thailand and describes a wide range of aspects about EEHV infection, including genetic variation and epidemiological aspects including prevalence, risk factors, and transmission. It is anticipated this study may potentially be used as a model for EEHV studies in the larger scale of Thailand and also other Southeast Asian countries, where the captive housing and husbandry situation is similar. In addition, the findings from this study will provide Thailand and the international elephant conservation community with baseline information regarding the threat of EEHV HD in Southeast Asia, particularly Thailand; and contribute to disease prevention programmes and monitoring protocols to better manage this disease in captive and free-ranging Asian elephants in both the immediate and long-term future.
1.2 The Asian elephant

The Asian elephant is considered as ‘umbrella and keystone species’ within their ecosystem, given that their foraging area is relatively large and that, during foraging, they clear vegetation for other species to forage; while acting as important seed dispersers, with seed distributed and fertilised within their faecal bolus (Dierenfeld, 2006). Therefore, reduction or loss of elephant populations will likely have a significant impact on the ecosystem. Asian elephants are also described as an ‘iconic cultural species’ in several countries, where they play a significant role in the culture, traditions, and daily lives of local communities. Given the significance of Asian elephants both ecologically and culturally, their conservation in the wild and captivity is critical; and a solid understanding of their biology, habitat range, conservation status, and threats is needed to help develop and implement effective conservation management. A brief overview of Asian elephants, in relation to conservation, is provided below.

1.2.1 Biology

Elephants belong to the order Proboscidea. Only three species of elephants remain extant: two species of African elephants (the African forest elephant and the African bush elephant); and one species of Asian elephant (Fig. 1-1) (Shoshani & Eisenberg, 1982).
When undertaking health assessments for conservation management of Asian elephants, there are several aspects of biology to consider. These include general appearance, behaviour, and reproductive status. Consideration of general appearance typically includes assessing body condition and external appearance, which includes ensuring the animal is free from injuries, looks bright and alert (with tail and ears flapping), and that the skin is clear from any external parasites (Mikota, 2006a).

Behaviour is also important to consider during health assessments, and can be a strong indicator of health status. Elephants are mega-herbivores, requiring large amounts of food per day (8–12% of their body weight, or 200–400 kg/day for Asian elephants) (Dumonceaux, 2006). They spend most of their time foraging and feeding, around 16–20 hours per day (Dumonceaux, 2006); and spend few hours sleeping, generally between 11pm–3am, when they lie down in lateral recumbency, although they may also rest in a standing position during the day. Juvenile elephants usually sleep more frequently
(generally more than once a day) and also have more play time (Schulte, 2006). Any decrease in appetite, feeding time, amount of food ingested, or any increase/decrease in play activities and sleeping duration and pattern, may indicate abnormal behaviour requiring further investigation.

Reproductive health, which often correlates with overall health, is another important criterion for assessment; and to assess reproductive health, it is important to know baseline values. Generally, a newborn calf stays with its mother until weaning at around three years of age. Elephants reach puberty at seven to twelve years of age and are sexually mature by 10–15 years (Schulte, 2006). The oestrous cycle in healthy female Asian elephants is normally 13–17 weeks long, comprising 8–10 weeks for the luteal phase and 4–6 weeks for the follicular phase (Brown, 2006). The gestation period is approximately 20–22 months long. Elephants which fail to reproduce as expected should be monitored closely, to identify and address any health related issues. Some reproductive issues however are related to poor management. Management induced reproductive failure may occur due to factors such as housing a low number of bulls in the facility, low numbers of interactions between male and female elephants, low sperm quality due to improper nutritional management, and high numbers of nulliparous females (Schmitt, 2006).

1.2.2 Population and distribution

Asian elephants inhabit 13 countries in Asia (Fig. 1-2): India, Sri Lanka, Nepal, Bangladesh, Bhutan, Myanmar, China (Yunnan only), Thailand, Lao People’s Democratic Republic (PDR), Vietnam, Cambodia, Malaysia (Peninsular Malaysia and Sabah only), and Indonesia (Kalimantan and Sumatra only) (Choudhury et al., 2008).
The Asian elephant is the only surviving species of the genus *Elephas* (Fig. 1-1). Subspecies of Asian elephants are generally distinguishable from each other by their home ranges, and by morphological and genetic analysis. Three subspecies are recognised: i) *Elephas maximus maximus* (Sri Lankan elephant), which occurs in Sri Lanka; ii) *Elephas maximus indicus* (Indian elephant), which represents the largest subspecies and occurs on the Asian mainland, including Bangladesh, Bhutan, Cambodia, China, India, Laos, Malaysia, Myanmar, Nepal, Vietnam, and Thailand; and iii) *Elephas maximus sumatranus* (Sumatran elephant), which occurs on the Indonesian island of Sumatra (Shoshani, 2006; Choudhury et al., 2008). The Borneo elephant, which is found only on the island of Borneo (Indonesia), has recently been proposed as a new subspecies of Asian elephant (*Elephas maximus borneensis*) (Fernando et al., 2003). To be considered a new subspecies, the animal must be both genetically and morphologically
distinct. Although the Borneo elephant is genetically distinct from the other subspecies, a preliminary study on the morphological measurements of these elephants showed no significant difference between this subspecies and *E. maximus indicus* (Nurzhafarina et al., 2008). Therefore, this proposed new subspecies is still awaiting further detailed morphological analysis.

### 1.2.3 Conservation status and threats

The Asian elephant has been listed as ‘Endangered’ and overall populations continue to decline (IUCN, 2008). The total population of Asian elephants was estimated to be between 40,000–50,000 worldwide (Choudhury et al., 2008), with approximately half the total population in India, and other relatively large populations in Malaysia, Myanmar, Sri Lanka, and Thailand. In any ecosystem, declines in numbers of individual species will produce a wide range of direct and indirect effects, both on other species in the ecosystem and on the ecological system as a whole (Sinclair et al., 2006; Smith et al., 2009). Loss of particular species which play key roles in an ecosystem – such as elephants – will have particularly adverse effects on other species which share the same habitat, ecology, and environment. Given the Asian elephant’s conservation status, along with the continuing declines of remaining populations and their important role in their ecosystem, their conservation is a priority; and the International Union for Conservation of Nature (IUCN) has compiled a list of current threats, summarised below (Choudhury et al., 2008):
a) *Habitat loss, degradation, and fragmentation.* One of the major threats for elephant conservation is an ongoing loss of natural habitat, due to factors such as the expansion of urban areas and human populations. Habitat fragmentation is also important and may potentially drive populations to extinction, due to loss of genetic diversity through inbreeding (Leimgruber et al., 2003).

b) *Poaching and illegal trading.* The Asian elephant is listed in Appendix I of the Convention of the International Trade in Endangered Species (CITES). As such, commercial trade in wild-caught specimens is illegal, and any trade or movement requires export and import permits. Despite this, poaching remains relatively common, particularly for ivory and meat, and represents one of a major threat for Asian elephants (Sukumar et al., 1998).

c) *Human and elephant conflict.* The increase in human populations and associated expansion of agricultural and residential areas leads to encroachment on elephant habitat, which in turn leads to human-elephant conflicts (Hedges, 2006). Loss of habitat and food sources drive elephants to find new food sources, which can lead to crop damage and human injuries; frequently resulting in the elephants being killed or injured by humans.

d) *Disease.* Disease may be a major factor in elephant population declines, in situations where disease leads to direct mortality, as well as when disease contributes indirectly to morbidity and mortality, for example through reducing immune fitness (Mikota, 2006a). A few diseases that have been well described in elephants include tuberculosis, haemorrhagic septicaemia, tetanus, and EEHV HD. There is an urgent need for further study of elephant disease, in the context of elephant conservation.
1.2.4 Asian elephant conservation in Thailand

Thailand has one of the largest Asian elephant populations in the world (Choudhury et al., 2008), and the elephant is also a species of cultural significance in Thailand, once used as a symbol on Thailand’s flag (Fig. 1-3). The number of elephants in Thailand continues to decline (Lair, 1999) with only 6500–7500 estimated to remain (S. Mahasawangkul, National Elephant Institute (Thailand), personal communication, 2014). Of this total, around 3500–4000 are free ranging (Thitaram et al., 2015), and the remaining 3000–3500 live in captivity (Angkawanish et al., 2009).

Figure 1-3. The former flag of Thailand (1855–1916), which had a white elephant as a symbol – showing the importance of elephants to Thai culture (from: https://en.wikipedia.org/wiki/Flag_of_Thailand#/media/File:Flag_of_Thailand_1855.svg).

The major threat for wild elephants in Thailand is habitat destruction, alteration and fragmentation, due to human population growth and incursion into remaining elephant habitat. Habitat loss and fragmentation leads to elephant population decline through loss of food sources, and a decrease in connectivity of populations with corresponding loss of
genetic diversity (Hedges, 2006). Poaching is not currently a major issue in Thailand, as poaching has been policed intensively by forest rangers, and ivory trading has recently been officially banned (Stiles, 2009; DLA Piper, 2016). Current knowledge regarding disease in wild elephants in Thailand is limited, due to the difficulties of obtaining samples, limitations associated with political priorities, and difficulties obtaining funding. Most elephant health research in Thailand uses captive populations and attempts to extrapolate findings to wild populations.

Unlike the free-ranging Asian elephant population, Thailand’s captive elephant population is considered relatively sustainable at present (C. Thitaram, Excellence of Elephant Research and Education (Thailand), personal communication, 2015). However, reproductive problems and health issues are generally observed in captive populations (Angkawanish et al., 2009). Low fecundity, and reproductive difficulties associated with interactions between males and females in captive populations, may lead to unstable and declining population sizes in the future (Thitaram et al., 2009). Diseases, including EEHV haemorrhagic disease, are of increasing concern for captive elephant conservation managers. EEHV HD, in particular, presents a mortality threat for young elephants with associated risks for captive populations in terms of recruitment and population structure.

Captive Asian elephants remain an integral part of human work, culture, and tradition in Thailand. The first captive elephants were obtained and used by the military (Csuti, 2006); however, following this they were used as part of traditional ceremonies (Fig. 1-4). Later, they were used in large numbers to facilitate logging practices and transport of goods and people, and this continued until the late 1980s. In 1989, forest logging was banned by the Thai government, and this led to a dramatically reduced need for captive elephants (Phuangkum et al., 2005). Presently, most of Thailand’s captive elephants are used for
tourism. Elephant-based tourism has become very popular in Thailand, making a significant contribution to the country’s economy, and helping to sustain captive elephant populations (Phuangkum et al., 2005).

**Figure 1-4.** Uses of captive Asian elephants in traditions in Thailand.

As elephant tourism has grown in Thailand, there has been a concurrent increased focus on improving captive elephant management. Given the value of elephants, their owners and managers are typically motivated to ensure that their elephants survive, remain healthy and reproduce. The Thai government now provides elephant health services free of charge. Education and information are available regarding how to improve fertility, fitness, and genetic diversity, with particular advice about how to avoid inbreeding (Thitaram et al., 2010). Despite this increasing interest in captive population management, and despite research taking place associated with health and reproductive issues, there are still significant knowledge gaps regarding health and disease management of elephants in Thailand; highlighting the need for further studies.
1.3 Disease as a conservation issue, with particular reference to EEHV haemorrhagic disease

1.3.1 Disease-associated extinction

The current wildlife extinction rate is estimated to be 100–1,000 times higher than the natural background rate (IUCN SSC Specialist Groups, 2007), and disease is one of many factors driving the current extinction crisis. Disease was previously considered to play a small role in extinctions; however, reports of disease-associated extinctions are increasing, linked to environmental changes and increasing human impacts (Scott, 1988; Ostfeld et al., 2008; Smith et al., 2009; Thompson et al., 2010). Scientists and policy makers agree that the world’s climate is changing at almost unprecedented rate, and that global warming is occurring (Burgman & Lindenmayer, 1998; Hannah et al., 2002). The Asian region has been predicted to experience some of the most severe effects from climate change (IPCC, 2014), and this is likely to have major implications for biodiversity in the region, through alterations in pathogen survival rates, vector abundance, transmission patterns, and host susceptibility (Hannah et al., 2002). It is possible that many ecosystems presently suitable for particular wildlife species may be unable to support these species in the future (Hannah et al., 2002).

The threat posed by infectious diseases to the long-term survival prospects of endangered species has been well documented (Daszak & Cunningham, 1999; Daszak et al., 2000; Daszak et al., 2001). The potential for disease to drive population decline and species extinction is a possibility that cannot afford to be discounted, and one that must be addressed in any endangered species recovery plan. Disease not only reduces population size through direct disease-related deaths; it also reduces reproductive ability, immune
strength, and overall health, leading to increased susceptibility to other disease agents, predators, and other environmental threats (Wobeser, 2006; Smith et al., 2009; Botzler & Brown, 2014).

1.3.2 The implications of disease for Asian elephant conservation

Elephants share many infectious agents with other species (for example external and internal parasites, foot and mouth disease, rabies, tuberculosis, trypanosomiasis, and haemorrhagic septicaemia). The current increase in land use for agriculture is likely to lead to increased disease transmission between domestic animals and wildlife, including elephants (Michel et al., 2006; Childs et al., 2007). This risk is elevated as disease is often of increased severity when transmitted to novel hosts (Childs et al., 2007; Parrish et al., 2008). For example an outbreak of encephalomyocarditis in South Africa caused the death of 64 free-ranging adult African elephants in 1993. The outbreak was correlated with increasing numbers of rats (the reservoir hosts) in the area, linked to the expansion of human dwellings and human activities in the elephants’ range (Grobler et al., 1995). Increasing levels of reverse zoonosis have also been reported with *M. tuberculosis* transmission between human and elephants (Michalak et al., 1998; Stephens et al., 2013).

EEHV HD is a disease of particular conservation concern, given that it causes fatal haemorrhagic disease in young elephants, particularly Asian elephants. This disease occurs sporadically throughout the world, and has affected up to 24% of elephant calves born in North America and Europe in the past 20 years, with at least 32 cases confirmed in North America alone (Latimer et al., 2011; Hayward, 2012). Given the reported and potential impacts of this disease, effective disease prevention plans are needed to minimise elephant population declines. Effective preventative management plans require
knowledge regarding disease status and reliable monitoring methods, both of which will be further investigated in this thesis.

1.3.3 Herpesvirus infection and host immune response

Here, the infection was defined as the invasion and multiplication of microorganisms such as bacteria, viruses, and parasites that are not normally present within the body. An infection may cause no symptoms and be subclinical, or it may cause symptoms and be clinically apparent. Viruses are one of the most important infectious agents, with a large diversity of host species, including animals, plants, bacteria, and fungi (MacLauchlan & Dubovi, 2011a). More than 3,000 species of virus have been identified (Simmonds et al., 2017), and it is estimated there are at least 320,000 undiscovered viruses in mammalian species (Anthony et al., 2013). Of these, herpesviruses form a common group as they are found in most species of animals, including humans and elephants. In general, most herpesviruses only cause mild symptoms; the severity of herpesvirus infection depends on the particular species of virus, as well as the host susceptibility, for example the B-virus (Cercopithecine herpesvirus 1) causes mild or inapparent symptoms in macaques, but can cause severe brain damage or death in humans (Grinde, 2013).

In 2009, the International Committee on the Taxonomy of Viruses (ICTV) classified the order Herpesvirales into three families: Herpesviridae, which comprises viruses normally found in mammals, birds, and reptiles; Alloherpesviridae, comprising viruses found in amphibians and bony fishes; and Malacoherpesviridae, for viruses in molluscs (McGeoch et al., 2006). The detailed classification of the order Herpesvirales is presented in Appendix 1.
Information about the specific size and composition of a virus is required to enable the type of virus to be identified by electron microscopy, and also to identify the specific immune response. Herpesviruses are large, double-stranded DNA viruses, approximate 200 nm in diameter (Davison, 2010). The herpesvirus particle is spherical in shape and comprises four main components, including (Fig. 1-5):

i) **Core.** This contains the viral genome, which is a large, linear, double-stranded DNA genome encoding 100–200 genes.

ii) **Capsid.** The viral genome is wrapped in a protein cage called the capsid, which is an icosahedron of diameter 125–130 nm.

**Figure 1-5.** The general structure of herpesvirus. (Left) Illustration of virion; (G) genome, (C) capsid, (T) tegument, (E) envelope. (Right) The structure of the herpes virion under electron microscope (Davison, 2010; MacLauchlan & Dubovi, 2011d).
iii) **Tegument.** The capsid is encased in an amorphous protein layer, called the tegument. The tegument is normally distributed asymmetrically, and its thickness may vary depending on the location of the virion within the infected cell.

iv) **Envelope.** The tegument is wrapped in the lipid envelope, which contains several virus membrane glycoproteins, as well as some cellular proteins.

Herpesviruses are extremely successful colonisers, with most animal species harbouring at least one herpesvirus (Burrow, 1977). By establishing latency, these viruses avoid elimination by the immune system and have the ability to persist in a host for life, if the host survives the primary infection (Grinde, 2013). Current research indicates that host species have their own individual herpesvirus, which has co-evolved with the host species (Grinde, 2013). This means that herpesviruses are well adapted to avoid their hosts’ immune defence mechanisms (McGeoch et al., 2006), and to survive in the host throughout the host’s life with occasional reactivation. The latency site in the host varies with the type of herpesvirus, but the sites are mostly found in the white blood cells and nerve cells (McGeoch et al., 2006). The latency of the virus is regulated by the host’s immunity; the virus may be reactivated when the host’s immunity is compromised, such as from concurrent infection, other stress events, or trauma (Grinde, 2013). Reactivation results in a release of the virus at the primary infected site and recirculation of the virus in the bloodstream, often with mild to no symptoms (McGeoch et al., 2006). While most herpesvirus infections cause minimal symptoms, several of these viruses, including EEHV, can occasionally have fatal effects on the host. Some herpesviruses can also cross species barriers from wildlife reservoirs to result in significant mortality (Ehlers, 2008). For example, Malignant Catarrhal Fever (MCF), caused by the Alcelaphine Herpesvirus-1 (AlHV-1) and Ovine Herpesvirus-2 (OvHV-2), do not cause disease in carrier and
reservoir animals (wildebeest and sheep, respectively). However, upon transmission to cattle, deer, bison, and pigs; MCF, a lymphoproliferative disease, occurs and is usually fatal (Reid, 1992; Ehlers, 2008).

1.3.4 The EEHV infection and conservation management of Asian elephants

EEHV HD is a disease of concern for elephant conservation, given the virus causes severe fatal haemorrhagic disease in young elephants (Hayward, 2012). Based on genetic analysis, the virus has been assigned to the family *Herpesviridae*, but into a new genus of the *Betaherpesvirinae* subfamily, named *Proboscivirus*. EEHV infection occasionally results in fatalities in young elephants due to haemorrhagic disease, while adult elephants seem to carry the virus latently, and intermittently shed via the eye, trunk, and vaginal secretions (Stanton et al., 2010; Latimer et al., 2011; Hardman et al., 2012; Sariya et al., 2012). In addition, another herpesvirus reported in elephants, Elephant Gammaherpesvirus (EGHV; subfamily *Gammaherpesvirinae*), has also recently been found in skin nodules and secretions from healthy elephants (Ehlers et al., 2008; Wellehan et al., 2008; Latimer et al., 2011). While EEHV can cause fatal haemorrhagic disease, EGHV has not been found to be pathogenic (Ehlers et al., 2008; Wellehan et al., 2008; Latimer et al., 2011). All known herpesviruses in elephants are listed in Table 1-1.
**Table 1-1. List of herpesviruses in elephants, with host preference and disease severity.**

<table>
<thead>
<tr>
<th>Herpesvirus</th>
<th>Host species</th>
<th>Pathogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Betaherpesvirus</strong> (Elephant Endotheliotropic Herpesvirus)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- EEHV1A</td>
<td>Asian elephant</td>
<td>Haemorrhagic disease</td>
</tr>
<tr>
<td></td>
<td>African elephant</td>
<td>Skin lesion</td>
</tr>
<tr>
<td>- EEHV1B</td>
<td>Asian elephant</td>
<td>Haemorrhagic disease</td>
</tr>
<tr>
<td>- EEHV2</td>
<td>African elephant</td>
<td>Haemorrhagic disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pulmonary nodule</td>
</tr>
<tr>
<td>- EEHV3</td>
<td>Asian elephant</td>
<td>Haemorrhagic disease</td>
</tr>
<tr>
<td></td>
<td>African elephant</td>
<td>Pulmonary nodule</td>
</tr>
<tr>
<td>- EEHV4</td>
<td>Asian elephant</td>
<td>Haemorrhagic disease</td>
</tr>
<tr>
<td>- EEHV5</td>
<td>Asian elephant</td>
<td>Haemorrhagic disease</td>
</tr>
<tr>
<td>- EEHV6</td>
<td>African elephant</td>
<td>Haemorrhagic disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pulmonary nodule</td>
</tr>
<tr>
<td>- EEHV7</td>
<td>African elephant</td>
<td>Pulmonary nodule</td>
</tr>
<tr>
<td><strong>Gammaherpesvirus</strong> (Elephant Gammaherpesvirus)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- EGHV1</td>
<td>Asian elephant</td>
<td>Subclinical infection</td>
</tr>
<tr>
<td>- EGHV2</td>
<td>Asian elephant</td>
<td>Subclinical infection</td>
</tr>
<tr>
<td>- EGHV3A</td>
<td>Asian elephant</td>
<td>Subclinical infection</td>
</tr>
<tr>
<td>- EGHV3B</td>
<td>African elephant</td>
<td>Genital lesions</td>
</tr>
<tr>
<td>- EGHV4</td>
<td>African elephant</td>
<td>Subclinical infection</td>
</tr>
<tr>
<td>- EGHV5</td>
<td>Asian elephant</td>
<td>Trunk nodules</td>
</tr>
</tbody>
</table>
1.3.5 Taxonomy and genetic structure of EEHV

EEHV belongs to the genus *Proboscivirus*, in the subfamily *Betaherpesvirinae* (Richman & Hayward, 2012). Unlike the other members of *Betaherpesvirinae*, the EEHV genome encodes ribonucleotide kinase B subunit (RRB), thymidine kinase (TK), and UL9-like origin binding protein (OBP) (Ehlers et al., 2006; Ling et al., 2013). Thus, it has been suggested that all EEHV types should be assigned to a new subfamily – the *Deltaherpesvirinae* (Ling et al., 2013; Richman et al., 2014).

All attempts to culture EEHV have failed to date (Latimer et al., 2011; Long et al., 2015); thus, genetic information is usually obtained from tissues and blood samples sourced from clinical cases. Analysis of the whole genome sequence of EEHV1 identified 177,136-bp, consisting of 115 open reading frames (ORF), including 37 conserved core genes common to all herpesviruses, 15 genes in common with some other herpesviruses, 3 core genes that are normally missing in Betaherpesviruses (RRB, TK, OBP), and 60 novel ORFs not found in any other herpesviruses (Ling et al., 2013). As such, the molecular biology, mechanism of latency, and pathogenesis are expected to be different than in any other herpesvirus subfamilies (Hayward, 2012).

Currently, seven types of EEHV have been classified, based on their genetic information. These types can be broadly divided into two main branches: the guanine-cytosine (GC) and adenine-thymine (AT) rich branches (Fig. 1-6). Although EEHV generally causes severe haemorrhagic signs in young elephants, there is variation in host preference and pathogenicity, according to EEHV type (Table 1-1). The type most responsible for causing fatal disease in Asian elephants is type 1A.
**Figure 1-6.** Genetic differentiation between the seven known types of EEHV, presenting two main branches; the GC-rich and AT-rich (Richman & Hayward, 2012).

### 1.3.6 History of herpesvirus infection in elephants

Initial reports of herpesvirus infection in elephants suggested the infection was non-pathological. Based on ultrastructure, herpesvirus-like particles and inclusion bodies were first found in the early 1970s, in the pulmonary nodules of asymptomatic wild African elephants (McCully et al., 1971). No illness was observed in these cases and no associated systemic signs were reported. In 1986, nodular skin lesions were found in a group of healthy African elephants, which had been transported from Zimbabwe to the USA. Intranuclear inclusion bodies were found consistently in skin lesions, without any further development of clinical signs (Jacobson et al., 1986). Similar herpesvirus-related warty skin lesions were also reported in Asian elephants, without any further laboratory confirmation (Pilaski et al., 1999).
The first fatal herpesvirus infection in elephants, reported in 1990, involved the death of a three year old female captive Asian elephant in Switzerland (Ossent et al., 1990). Extensive haemorrhage was found on post-mortem examination. Basophilic intranuclear inclusion bodies were found under light microscopy of myocardial and hepatic cells. Electron microscopy revealed enveloped herpesvirus-like particles approximately 120 nm in diameter. Viral isolation failed and no molecular diagnosis was available at that time (Ossent et al., 1990). Given the absences of specific diagnostic tests, a serological test against Bovine Herpesvirus (BHV) types 1, 2, and 4 was initially conducted (Metzler et al., 1990). Testing was performed on a pericardial fluid sample obtained from the dead baby elephant, as well as serum samples from her herd mates and elephant serum samples sourced from elsewhere. Results indicated the serum were weakly positive against BHV2. However, BHV had not been found outside Artiodactylids, suggesting that the elephants were possibly infected with a herpesvirus that was immunologically related to BHV2, and was potentially widely distributed among captive Asian elephant populations (Metzler et al., 1990). In 1997, similar serological surveys were conducted on healthy Asian elephant populations in South India. A total of 109 serum samples were collected and tested for BHV1 and BHV2, using a passive haemagglutination (PHA) test. Unexpectedly, 23% of them were seropositive to BHV2, and 4% were positive to BHV1. This supported the hypothesis that Asian elephants may be reservoirs for herpesviruses that are serologically related to BHV1 and BHV2 (Bhat et al., 1997).

Despite multiple attempts of definitive diagnosis, the virus remain unidentified. In 1995, the acute death of a 16 month old USA captive Asian elephant, followed by the similar deaths of an 11 month old captive African elephant in 1996 and a 17 month old captive Asian elephant in 1997, provided numerous diagnostic samples to pursue a diagnosis.
Each of these individuals presented with acute death without obvious preceding symptoms. However, in each case, gross lesions included extensive haemorrhage, and intranuclear inclusion bodies were found under microscopy (Richman et al., 1999). Given the failure of attempts to culture virus in any cell lines, a molecular-based diagnostic test was developed, which revealed a new pathogen closely related to the Betaherpesvirinae (Richman et al., 1999). This new pathogen was named ‘Elephant Endotheliotropic Herpesvirus’ (Richman et al., 1999; Richman et al., 2000a; Richman et al., 2000b).

Once the diagnostic test was available, retrospective studies were conducted on all stored tissue samples from the previous deaths of young elephants. Between 1983–1999, of the 34 Asian elephants born in captivity in North American zoos, seven were found to have died from EEHV HD, and another was found to have the virus but survived following intensive antiviral drug treatment (Richman et al., 2000b; Schmitt et al., 2000). The research showed that affected elephants were most likely to be young, ranging from 18 months to seven years (Richman et al., 1999; Richman et al., 2000b). No sex predilection was observed. Based on genetic analysis, two types of EEHV were identified, including EEHV1 – which appears to be pathogenic only in Asian elephants – and EEHV2, which is pathogenic only in African elephants (Richman et al., 1999; Richman et al., 2000a; Richman et al., 2000b).

Of particular interest to researchers, the use of the molecular diagnostic tests on stored biopsy tissue samples from skin papilloma and vulva lymphoid patches from healthy captive African elephants revealed the presence of a virus with DNA sequences matching those of EEHV1, the EEHV type which in Asian elephants caused deadly haemorrhagic disease (Richman et al., 2000a; Richman et al., 2000b). At that time, EEHV1 had not been found outside of captive elephant populations in North America and Europe, where
the two species of elephants had been mixed together in captivity. EEHV1 was therefore hypothesised to have evolved, via cross-species transmission, from non-pathological infection in African elephants, to causing a frequently-fatal disease in Asian elephants (Richman et al., 1999; Richman et al., 2000a; Richman et al., 2000b).

Following the development of the molecular-based diagnostic test, many more cases of the disease were identified. In 2009, two young captive Asian elephants in North American zoos died within two and eight days following the first observation of clinical signs; and EEHV HD was suspected. Post mortem examination revealed diffuse haemorrhage in most internal organs. However, these two cases were negative for known EEHV types (EEHV1 and EEHV2). Intensive molecular tests were performed, and new types of EEHV were found which had genetically diverged (Garner et al., 2009). The new types were designated EEHV3A and 3B (Garner et al., 2009), which were later changed to EEEHV4 and EEHV3 respectively (Latimer et al., 2011). EEHV3 and 4 show less selective organ tropism than EEHV1 and 2, with the virus more likely to be distributed throughout the body, which was postulated to explain EEHV3/4’s virulence compared to the other types (Garner et al., 2009).

In addition to the four types of EEHV previously discovered, EEHV5 was detected in 2011, in routine blood tests from an adult healthy wild-born Asian elephant who presented with oral ulceration (Latimer et al., 2011). In 2013, an outbreak of EEHV5 infection (viremia) was detected in a herd of seven captive Asian elephants, after mild symptoms – including lethargy, temporal gland swelling, vesicles on the tongue, and oral mucosal hyperaemia – had been noticed in an adult (42 years old) female wild-born Asian elephant from the herd (Atkins et al., 2013). This individual had been treated with antiviral drugs until clinical signs and viral load level were resolved. During the period of treatment,
EEHV5 viral DNA was detected in the blood or trunk washes of all other members of the herd; however, none developed clinical signs (Atkins et al., 2013). Further, the first reported fatality related to EEHV5 infection was reported in 2012 (Denk et al., 2012; Wilkie et al., 2014), and involved a 20 month old captive male elephant calf which had a history of developing oral and peri-anal lesions throughout its life, and was ill for six days prior to euthanasia. A post-mortem examination revealed extensive widespread haemorrhage, including the meninges. Intranuclear inclusion bodies were present in most internal organs, including the adrenal gland and oral mucosa; and molecular tests revealed and confirmed EEHV5 infection (Wilkie et al., 2014).

In addition to types 1–5, EEHV6 has been discovered from blood samples from a 15 month old female African elephant which developed signs of limb stiffness, but which survived following treatment with antiviral drugs (Latimer et al., 2011). The first fatal case associated with EEHV6 infection was reported to be a captive juvenile African elephant in Thailand (Kongmakee et al., 2015). The most recently identified EEHV type is type 7, which was found in the lung nodules of an asymptomatic captive adult African elephant in a North American zoo (Zong et al., 2015). No fatal cases related to EEHV7 infection have been reported to date.

To date, at least 60 cases of clinical EEHV infection have been reported in North American and European zoos (based on gross lesions, histopathological results, and molecular evidence), with 80–85% fatality rate among infected individuals (Hayward, 2012; Richman & Hayward, 2012). As noted above, the disease often but not always occurs in young elephants (under 15 years old), with most cases reported in juveniles (between the ages of one and five years old). Treatment of the disease is challenging with
rapid progression, and only ten survivors have been reported following aggressive treatment with antiviral drug and supportive care (Hayward, 2012).

Although Elephant Endotheliotropic Herpesvirus has been studied and monitored intensively in captive elephants in North America and Europe, only limited information about this disease has been obtained from Asia, including from the Asian elephant’s natural range countries. The first confirmed case of fatal EEHV infection in Asia was reported in Cambodia in 2006 (Reid et al., 2006). A three year old female wild-born Asian elephant, which had been in captivity since six months old, died suddenly without any observed symptoms or treatment. PCR-based diagnostic tests confirmed an infection which matched EEHV1 infection (Reid et al., 2006). In 2001, the first attempts were undertaken to screen for EEHV in healthy elephant populations in Asia (Hildebrandt et al., 2005). Whole blood and biopsy tissues from retropharyngeal lymph nodes were collected randomly from captive Asian elephants in Thailand. The samples were tested for EEHV using conventional PCR techniques. Although none of the samples tested positive for EEHV1 at that time (Hildebrandt et al., 2005), both EEHV1 and EEHV4 were later reported (in 2013) to have caused fatal haemorrhagic disease in two young Asian elephants in Thailand (Sripiboon et al., 2013). This was also the first report of EEHV4 outside North America and Europe.

The focus of EEHV researchers and elephant conservation managers widened to India in 2013, following reports of the first occurrence of EEHV HD in South India (Zachariah et al., 2013). The report confirmed the occurrence of disease in both wild and wild-born captive elephant populations. EEHV1 was found in nine out of 15 dead young elephants tested. Extensive genetic diversity was found between cases, and most of the positive cases comprised independent unrelated strains. This finding suggests that EEHV1A and
1B, at least, are likely to be natural endogenous pathogens which have coevolved with
Asian elephants for long periods of time, and the occurrence of disease is more likely to
be sporadic, rather than epidemic (Zachariah et al., 2013). As surveillance technologies
have developed, EEHV surveillance in Asia has been undertaken in South India, in both
captive and semi-captive Asian elephant populations, using real-time PCR methods
(Stanton et al., 2014). The 2014 study included a total of 46 healthy Asian elephants, for
which trunk wash samples were collected and tested for the presence of all types of
EEHV. Results revealed 35% prevalence of subclinical EEHV infection in the sample
group. The EEHV type with the highest reported prevalence was EEHV5, despite the fact
that no fatalities associated with EEHV5 infection have been reported in South India to
date. The high reported prevalence of EEHV5 infection, and lack of associated fatalities
suggests that EEHV5 may has low pathogenicity or, has a well-adapted host-pathogen
relationship. In addition, fatal EEHV infection has also been confirmed in several of the
Asian elephant’s other range countries, including Lao PRD (Bouchard et al., 2014),
Indonesia (M. Wahyu, Veterinary Society for Sumatran Wildlife Conservation
(Indonesia), personal communication, 2015), and Myanmar (Long et al., 2015).

1.3.7 Pathology of EEHV HD in Asian elephants

Pathological (clinical, gross, and histopathological) changes are generally used to support
differential diagnoses, or are used as evidence for an early diagnosis. Abnormal
behaviour, clinical signs and blood profiles, are generally used as indicators for early
detection of EEHV HD, as the molecular diagnostic test to confirm EEHV infection
usually takes at least 3–4 hours, which given the rapid progression of the disease is often
too long to enable timely treatment. Clinical, gross, and histopathological findings for
EEHV HD are unique, and are presented below.
Clinical pathology

The disease progresses quickly, in many cases only a few general clinical signs – such as lethargy, dullness, and anorexia – are observed before death (Richman et al., 2000a; Richman et al., 2000b). Therefore, daily behavioural observation and daily body temperature measurement should be conducted in every young elephant. If an animal shows abnormalities in behaviour and body temperature, then action should be taken. Given the virus’ tendency to remain in the endothelial cells and cause vascular damage, other common clinical signs may include head and facial swelling, as a result of subcutaneous oedema (Richman et al., 2000a); and as the disease progresses, the vascular damage can lead to tongue cyanosis and petechial haemorrhage at the tip of the tongue (Richman et al., 2000a; Richman & Hayward, 2012). Other clinical signs occasionally observed include oral ulceration, lameness, abdominal discomfort, and temporal gland swelling (Garner et al., 2009; Richman & Hayward, 2012; Atkins et al., 2013). However, these signs are relatively late in onset (six to twelve hours following behavioural change) and if using these as initial signs, the veterinarian is likely to miss the window of opportunity to treat successfully.

Changes in haematological parameters are not consistent between cases. A leukopenia and decrease in erythrocyte counts are often seen, but not in every case (Cracknell, 2008; Richman & Hayward, 2012). Decreased thrombocyte counts are reported to be a useful diagnostic parameter, as there is a trend towards severe thrombocytopenia in most cases (P. D. Ling, Baylor College of Medicine (USA), personal communication, 2015). Biochemistry values do not change significantly during infection (Richman and Hayward, 2012). Hypoalbuminaemia can be observed, associated with protein loss from vascular damage (Cracknell, 2008). Limited information about blood gas analysis has been
reported; in one case report, respiratory acidosis was recorded despite intranasal oxygen being given (Cracknell, 2008).

**Gross pathology**

EEHV is endotheliotropic, preferring to live in endothelial cells, therefore the hallmark of this clinical infection is ‘extensive haemorrhage’ (Richman et al., 2000a; Richman et al., 2000b). The presence of virus in the endothelial cells contributes to vessel damage, leading to widespread haemorrhage throughout the body (Richman & Hayward, 2012). Rapid death is likely due to organ failure, especially heart failure, and hypovolemic shock due to fluid leakage through vascular damage. Retrospective studies of cases in North America between 1983–1999 showed general gross pathological findings as follows (Richman et al., 2000a; Richman et al., 2000b):

- Extensive petechial to ecchymotic haemorrhage involving the epi-, endo-, and myocardium (Fig. 1-7), and/or pericardial effusion
- Diffuse petechial haemorrhage in the serous membranes of internal organs
- Oral, laryngeal and large intestine ulcers
- Hepatomegaly
**Figure 1-7.** Extensive haemorrhage of the heart is the hallmark of gross pathological finding for EEHV haemorrhagic disease (Cracknell, 2008).

**Histopathology**

Histopathological findings are one of the key components required for disease confirmation, as the gross pathological lesions may appear similar to those of other diseases. As described above, the virus displays endotheliotropism; therefore with regard to histopathology, amphophilic to basophilic intranuclear inclusion bodies (INIB) can be found in the endothelial cells of the capillaries in the myocardium (Fig. 1-8a), tongue muscle, and hepatic sinusoid of the liver (Richman et al., 1999; Richman et al., 2000b). Electron microscopic examination reveals virus particles 80–92 nm in diameter which is morphologically related to herpesvirus particles (Richman et al., 1999; Richman et al., 2000a; Richman et al., 2000b) (Fig. 1-8b). Extensive haemorrhage is also commonly found in the heart tissue, with mild infiltrate of lymphocytes, monocytes, and neutrophils between myofibers (Richman et al., 2000a).
Figure 1-8. The histopathological findings of EEHV HD. (a) Intranuclear inclusion body in a capillary endothelial cell of the heart under light microscope (arrow). (b) The viral particle under electron microscope (arrow) (Richman et al., 1999).

1.3.8 Diagnostic tests for EEHV infection

To definitively diagnose EEHV infection, molecular tests are required; as the pathological changes described are also consistent with other diseases, such as endomyocarditis virus infection and haemorrhagic septicaemia. As the virus has not been able to be grown in any cell lines to date (Latimer et al., 2011; Long et al., 2015), molecular-based techniques (PCR) are at present the most commonly used diagnostic methods for diagnosing EEHV. In addition, serological tests (ELISA) for detecting antibodies against EEHV infection have also recently been developed (van den Doel et al., 2015). All current EEHV diagnostic tools are listed below:
Conventional PCR

Conventional PCR (cPCR) is currently the most common technique used for EEHV confirmation, as nucleotide sequences for the virus can be obtained using this method. Conventional PCR involves a reaction in which the target nucleotide sequence is repeatedly amplified and visualised on gel electrophoresis. The first conventional PCR to detect EEHV infection was developed and documented in 1999 (Richman et al., 1999). Through this method, two types of EEHV (EEHV1 and EEHV2), were identified. Subsequently, two other types of EEHV (EEHV3 and EEHV4) were discovered, and then the type-specific primer pair was developed (Garner et al., 2009). As the likelihood of discovering new subtypes of EEHV increased, a redundant primer (based on polymerase gene locus) was developed which could detect most types of EEHV (Latimer et al., 2011). This redundant universal primer was widely used in standard protocols for screening EEHV infection. However, as it is redundant, a specific primer for each type of EEHV has also been developed, to obtain detailed genetic information and increase the sensitivity and specificity of the reactions (Latimer et al., 2011).

Quantitative real-time PCR

Quantative real-time PCR (qPCR) was developed to enhance the capacity and efficiency of EEHV diagnostic tests (Stanton et al., 2010; Stanton et al., 2012). The benefits over real-time PCR include being able to detect low levels of virus during shedding periods; being able to detect the virus at an early stage; and being able to determine the dynamics of the virus – information which could be used to more effectively monitor treatment outcomes. Previous research has shown that this method can detect low levels of the virus being shed from healthy elephants via trunk wash, conjunctival swab, vaginal swab, and
routine blood sampling (Stanton et al., 2010; Hardman et al., 2012). This method is, therefore, the most preferable to use as a surveillance tool in healthy populations, where virus numbers tend to be low.

**ELISA**

Enzyme-linked immunosorbent assay, or ELISA, is a method usually used to measure the levels of antibody directed against the pathogen of interest. The primary obstacle to the development of an ELISA to measure antibody levels against EEHV infection is the fact that the virus has, to date, not been cultured; therefore, the properties of the viral antigenic sites are not well understood. However, based on the known properties of other herpesviruses, glycoprotein B (an envelope protein known to induce virus neutralising antibodies in other herpesvirus infections) has been used to develop an ELISA for EEHV (Fickel et al., 2003; van den Doel et al., 2015). Recent research has reported approximately 80% correlation between antibody levels and infection history (van den Doel et al., 2015). These findings indicate the potential to use this tool to evaluate disease prevalence and herd seroprevalence.

**1.3.9 Treatment of clinical EEHV infection**

As EEHV cannot, as yet, be isolated in the laboratory, vaccine development has been challenging, with no success to date (Bennett et al., 2015b; Long et al., 2015) Treatment for EEHV haemorrhagic disease is therefore based on the use of antiviral drugs used in human medicine, in conjunction with intensive supportive treatment. Survival rates associated with EEHV HD are low and depend on several factors, including virus strain, individual immunity level, and the onset of treatment intervention (Zong et al., 2007;
Hayward, 2012; Richman & Hayward, 2012). Given the rapid progression of the disease, early treatment significantly increases the chances of survival (Wiedner et al., 2012). It is recommended that treatment should commence as soon as possible after initial clinical signs are observed, even before confirmation is obtained from the laboratory. Given that early treatment is imperative, frequent monitoring for signs of infection is also crucial; which highlights the importance of developing and maintaining effective monitoring protocols.

Antiviral drugs usually used in human medicine, which have been used successfully to treat clinical EEHV infection include famciclovir, ganciclovir, and acyclovir (Schmitt et al., 2000; Cracknell, 2008; Wiedner et al., 2012). The first, famciclovir, is an anti-herpesvirus drug which is used in the treatment of Herpes Simplex Virus (HSV) in humans. However, this drug needs to be transformed to its active form (penciclovir) using thymidine kinase. Although research has shown EEHV possesses the thymidine kinase gene (Ehlers et al., 2006), the gene’s functionality has not been tested. The effectiveness of famciclovir therefore remains unclear. The active form of famciclovir, penciclovir, acts as an inhibitor for viral DNA synthesis, stops virus replication, and prevents the virus from spreading to other cells. However, the drug cannot assist already virus-damaged cells to recover; it only prevents further pathological damage – therefore, early treatment intervention remains essential (Cracknell, 2008). Use of famciclovir as a treatment for clinical EEHV infection is common, however difficulties with drug administration have been reported, as it requires either oral or rectal administration. Although the pharmacokinetics of oral famciclovir has been studied and reported in Asian elephants (Brock et al., 2012), when using oral famciclovir during clinical EEHV infection, the viral loads continued to increase and there was likely a lag effect whilst drug levels increased.
to therapeutic levels, so this drug was ineffective in clinical cases (Stanton et al., 2013). The two remaining drugs, ganciclovir and acyclovir, can both be given intravenously, however, neither the pharmacokinetics nor the pharmacodynamics of these latter two drugs has been studied in elephants (Wiedner et al., 2012).

In addition to treatment with antiviral drugs, supportive treatment is imperative and must be conducted intensively. Fluid therapy is essential, as fluid will be lost from vascular components (due to vascular damage) into the peripheral tissues, which can potentially lead to hypovolemic shock (Cracknell, 2008). Analgesia is recommended, to help prevent inflammation from peripheral oedema and haemorrhage. Antibiotics are also usually given, to prevent secondary bacterial infection. Intranasal oxygen should also be supplied to support the cardiovascular system (Cracknell, 2008; Wiedner et al., 2012).
1.4 Aims of the project

Research to date on EEHV has highlighted potentially severe implications for elephant conservation, owing to the severity of the disease, particularly for young individuals (Hayward, 2012; Richman & Hayward, 2012). Asian elephants play an important role in Southeast Asian ecosystems, and they are also an integral part of people’s lives, traditions, and cultures. This study aimed to investigate the status of EEHV infection, both clinical and subclinical, in captive Asian elephants in Thailand, which has one of the largest Asian elephant populations in the world. To obtain a better understanding of the epidemiology of EEHV HD in Thailand, I conducted a molecular genetic study which evaluated the disease epidemiology based on genetic information. Most of the previous EEHV HD–suspected cases in Thailand were re-examined and tested for EEHV infection using multiple EEHV gene-specific loci analysis. Genetic information from all positive cases was then compared among cases, as well as with other previously-reported cases across the world.

To obtain information on the status of EEHV infection in asymptomatic Asian elephants, I developed a new diagnostic tool, based on the SYBR Green I-based real-time PCR technique. Samples were collected from healthy Asian elephant populations across Thailand and tested for the virus. The prevalence of subclinical EEHV infection and variables associated with EEHV detection were evaluated and reported. Given the virus is generally shed intermittently, I also conducted a longitudinal study, which aimed to evaluate the viral shedding pattern as well as factors associated with viral activation, reactivation and infection. Faecal samples were also collected, and the concentrations of glucocorticoid hormone were analysed to determine if there were any stressful events
occurring at that time. In addition, serological tests (ELISA) against the EEHV infection were conducted, and the results were related to the presence of EEHV using qPCR testing.

This study provides an important overview of clinical and subclinical EEHV infection in Thailand, including risks and implications of EEHV HD for elephant conservation. It is hoped that the study’s findings may be used to develop effective recommendations and guidelines for EEHV monitoring and management to aid Asian elephant conservation management in Thailand and other countries in Southeast Asia. This research may also serve as a model for other EEHV-related projects in Thailand and other countries within the Asian elephant’s range. Moreover, this study attempts to bridge significant gaps in our knowledge of the epidemiology of EEHV infection in Thailand. It is hoped that these research findings will benefit Asian elephant conservation, help to protect a national and cultural icon, and safeguard traditional ways of life and employment in human communities throughout the region.
1.5 Organisation of chapters

This thesis comprises seven chapters. Chapter 1 provides general information relating to elephants and herpesviruses, and presents the study’s aims and objectives. Chapter 2 presents the general study methods. Chapter 3 presents and discusses the results from the first part of the study: a retrospective analysis of most of the EEHV HD-suspected cases occurring in Thailand, testing for the presence of EEHV, analysis of genetic information, and evaluation of molecular epidemiological relatedness. Chapter 4 presents the development of a new EEHV diagnostic method using SYBR Green I-based quantitative real-time PCR, and discusses its application to field studies. Chapter 5 presents and discusses the results from the second part of the study: an investigation of the prevalence of subclinical EEHV infection in healthy Asian elephants in Thailand, using a cross-sectional study design, to determine the overall status of subclinical EEHV infection in Thailand. Chapter 6 presents and discusses the results of a longitudinal study where samples have been collected from an EEHV-positive herd, comprising of an EEHV HD survivor and its herd mates. The pattern of EEHV shedding detected through qPCR analysis is compared to EEHV serological status, and the association between the two diagnostic assays is analysed. The concentrations of glucocorticoid hormone in faeces are also used to monitor stress levels during this study period. Variables associated with EEHV activation and shedding are analysed and reported. Chapter 7 summarises the study overall and discusses the implications of the study’s findings for future EEHV research, disease management, and elephant conservation in Southeast Asia.
CHAPTER 2

GENERAL METHODS
This chapter describes the project’s study sites, general sampling techniques, and general diagnostic tests. Specific techniques and laboratory procedures which are associated with particular aspects of the study will be described in the relevant chapters.

2.1 Study sites

This project focused on investigating the status of clinical and subclinical EEHV infection in one of the Asian elephant’s range countries – Thailand. Thailand is located in the middle of mainland Southeast Asia, and comprises around 510,000 km$^2$ of land area. The human population is estimated to be around 67 million people (The World Bank, 2014). Thailand is divided into six main regions; northern, north-eastern, central, eastern, western, and southern Thailand.

Thailand has the fourth largest population of Asian elephants in the world (Choudhury et al., 2008). The current population of Asian elephants in Thailand is approximately 6500–7500, including both wild and captive elephants. The wild Asian elephant population is estimated to be around 3500–4000 animals (Thitaram et al., 2015), while the captive population is estimated to be around 3000–3500 (Angkawanish et al., 2009). Captive Asian elephants include zoo elephants, elephants kept for tourism activities, working (logging) elephants, and rescued elephants in sanctuaries. The majority of Asian elephants in captivity in Thailand are those kept for tourism.

Given the challenges associated with the collection of samples in asymptomatic elephants for EEHV diagnosis, particularly the need for elephants to be either well-trained or under physical restraint, this study focussed primarily on captive rather than wild elephants for
sample collection. The estimated number of captive Asian elephant populations in Thailand in each region is presented in Fig. 2-1.

**Figure 2-1.** Based on geographical location, Thailand can be divided into six main regions. The estimated number of captive Asian elephants in each region is indicated, showing that the main populations of captive Asian elephants in Thailand live in the north-eastern, northern, and southern parts of the country.
Thailand was chosen as the focus of this study not only because of its status as a country with one of the largest remaining populations of Asian elephants, but because EEHV HD has been reported and confirmed in Thailand, with more than 10 cases of clinical EEHV infection reported since 2006 (Sripiboon et al., 2013; Lertwatcharasarakul et al., 2015; Sripiboon et al., 2015). Thailand is also important as a focal country because, despite these reports of infection, no intensive EEHV research or large-scale disease surveillance have been conducted in Thailand.

For the retrospective part of the study (Chapter 3), samples were obtained from all four EEEV diagnostic facilities in Thailand: i) the Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen Campus, NakornPrathom (FVM-KU-KPS); ii) the Faculty of Veterinary Science, Mahidol University, NakornPrathom (FVS-MU); iii) the Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai (FVM-CMU); and iv) the Veterinary Research and Development Center, Lower North-Eastern region, Surin (VRD-LNE) (Fig. 2-2).

Sampling sites for the cross-sectional study were distributed across the country, and details of specific locations are described in Chapter 5. While initial sample size calculations for the cross-sectional study (Chapter 5) were undertaken using Epitools (Sergent, 2015), the on ground situation and the need for sampling to be undertaken in conjunction with a government veterinarian meant that sample size was determined associated with access and collaboration with government veterinarians (see details in Chapter 5). Sampling for the longitudinal study focused on a captive elephant facility in northern Thailand (18.250683, 99.2220751), which had a confirmed case of clinical EEHV infection (see Fig. 2-2). Specific details of this EEHV-positive herd are described in Chapter 6.
Figure 2-2. Locations of all four EEHV diagnostic facilities in Thailand from which samples were obtained for this project’s retrospective research: (a) FVM-KU-KPS, (14.02081, 99.97322); (b) FVS-MU (13.79819, 100.31711); (c) FVM-CMU (18.7588, 98.94024); (d) VRD-LNE (14.77529, 103.43654). The (*) indicates the sampling site for a longitudinal study (Chapter 6).
2.2 Sample collection and sampling regime

Previous research has reported that EEHV could be detected in several types of samples, including blood, tissue samples, trunk wash, conjunctival swabs, trunk swabs, and lesion biopsy and swab (Schaftenaar et al., 2010; Stanton et al., 2010; Latimer et al., 2011; Hardman et al., 2012; Sariya et al., 2012). The general sample collection protocols used in this study are described below.

To investigate the presence of EEHV in clinical cases, this study tested whole blood and tissue samples of elephants that were submitted to the four EEHV diagnostic facilities in Thailand, during 2006 to 2014. All of the tissue samples were from elephants that were suspected to have died from disease associated with EEHV infection, and whole blood samples were obtained from suspected clinical cases. A total of 22 EEHV-suspected fatality cases, and two EEHV-suspected clinical cases were included in this study. The EEHV report form used in this study to collected EEHV-related information is presented in Appendix 2.

To survey and monitor subclinical EEHV infection in healthy Asian elephant populations, whole blood samples, conjunctival swab, and trunk swab were collected for this study. We aimed to collect the samples from all age classes, which were defined according to the criteria from Arivazhagan and Suumar (2008); baby (newborn to one year old), juvenile (1-5 years old), sub-adult (5-15 years old), and adult (more than 15 years old). In addition to these samples, semen samples were collected using manual collection technique from nine individual bull elephants, to investigate the possibility of EEHV transmission via semen (see Chapter 4); and fresh faecal samples, which were serially
collected for hormonal analysis, were collected from fresh faecal bolus of five individual elephants in the EEHV-positive herd (see Chapter 6).

All collection procedures were approved by Murdoch University’s Animal Ethics Committee (Permit No. R2582/13), and by the owners of the individual elephants. Samples were only collected from elephants that were trained to facilitate sample collection, using standard training techniques based on positive reinforcement. No elephants were sampled if they had not been trained to enable sample collection, or were non-compliant during the sampling attempt. Any animals which demonstrated a reluctance to participate or cooperate with a particular sampling protocol were excluded from sampling.

2.2.1 Tissue samples

Tissue samples available for this research included samples which had been collected from most of the dead elephants suspected to have died of disease associated with EEHV infection in Thailand. Full necropsy procedures, including gross pathological and histopathological examinations, had been performed on only 12 out of 22 suspected EEHV HD cases. Necropsy protocols followed the standard guidelines for elephant necropsies established by the American Zoo and Aquarium Association (http://www.elephantcare.org/protodoc_files/2012/eleph_research_and_necropsy_protocol_august_2012.pdf). For the remaining 10 deceased elephants, a single sample (generally tongue) was collected without full necropsy examination. Details of general gross and histopathological findings of cases of EEHV fatalities are described in Chapter 1, Section 1.3.7.
EEHV-confirmed cases typically involve extensive petechial to ecchymotic haemorrhage in internal organs, including the heart, liver, tongue, and intestinal tract, which are particularly collected for EEHV diagnoses using molecular techniques. Samples were collected as soon as possible after death, to minimise autolysis and / or environmental contamination, which could impact on molecular diagnosis. Following post-mortem examination, fresh tissue samples of target organs or lesions minimally 2 cm x 2 cm in size were required for molecular diagnostic tests. These tissue samples were kept in individual sterile plastic bags and stored on ice in an esky (at approximately 4°C) during transportation and then at -20°C/-80°C for longer periods of storage prior to use for diagnostic testing.

2.2.2 Whole blood samples

Whole blood samples were obtained for this study from both clinically ill and healthy elephants. Elephants were trained or restrained physically under positive reinforcement during blood collection. Blood was collected from the auricular vein on either side of the ears (Fig. 2-3). An 18 G or 20 G needle, connected to a 5 ml syringe was used to withdraw blood. Isopropyl alcohol (70%) was applied to the sample site prior to collection, in order to reduce the likelihood of infection or contamination. At least 2 ml of whole blood was stored in an Ethylenediaminetetraacetic acid (EDTA) coated tube, as required for later extraction of DNA, and subsequently used in the polymerase chain reaction (PCR). Samples were kept on ice or 4°C during transportation and stored at -20°C/-80°C for long-term storage.
2.2.3 Swab samples

Previous studies have identified a range of swab samples that can be used for EEHV detection, including conjunctival swabs, trunk swabs, lesion swabs, palate swabs, and vestibule swabs (Schaftenaar et al., 2010; Hardman et al., 2012; Sariya et al., 2012). This study used both conjunctival and trunk swabs, which are the swab samples that can be collected safely from elephants. The swabbing protocols followed protocols established by previous researchers (Hardman et al., 2012), and involved soaking sterile cotton swabs in phosphate buffered saline (PBS) or sterile saline solutions, prior to swabbing the conjunctiva (Fig. 2-4) and nostrils. Care was taken during trunk swabbing to avoid any dirt within the nostril. In the case that elephants closed their eyes during sample collection, swabbing inside the eyelid while the eye was closed was considered sufficient.
Following sampling, the swabs were placed in a 1.5 ml microcentrifuge tube containing 250 µl of PBS solution, and the tube was kept cool (4°C) during transportation. Samples were processed as soon as possible, and always within 48 hours after collection. Processing involved the tube being centrifuged at 6000 x g for 3 minutes at room temperature. The swab was then discarded, and the supernatant was used in the later DNA extraction step.

**Figure 2-4.** Swabbing site for conjunctival swab.

### 2.2.4 Faecal samples

Faeces were collected during this project for hormonal analysis. Approximately 10 grams of fresh faecal samples were collected, following breaking and mixing of the faecal bolus. Faecal samples were collected at the same time of the day in order to avoid the effect of diurnal changing of stress hormones. The samples were kept in sterile plastic bags and stored at -20°C for later analysis.
To prepare faecal samples for hormonal analysis, approximately five grams of the faecal sample was placed in a plastic cup and incubated at 60°C for 24–48 hours, until dry. The sample was then left to cool to room temperature, during which time the grass content was removed from the dried faecal content and discarded. Two grams of the remaining faecal content was measured to use for hormonal extraction (details are described in Chapter 6, Section 6.2.5).

2.2.5 Semen samples

Semen collection was only undertaken by well-trained personnel, and samples were only collected from elephants trained for semen collection, to minimise adverse responses during collection. Bull elephants which had reached sexual maturity were selected and trained for semen collection. Semen samples were collected using manual stimulation techniques as previously described (Schmitt & Hildebrandt, 1998). Briefly, for this technique the elephant was restrained physically to prevent injuries to both the elephant and the person collecting the sample. Faeces were removed manually from the rectum, and the protrusion and erection of the penis were stimulated by the rectal massage at the ampulla area. Once the penis was protruded, sterile gauze was used to clean the penis to prevent environmental contamination. As the massage was continued, ejaculation occurred and the sample was collected by placing a collection sleeve over the end of the penis (Fig. 2-5). For this study, at least 500 µl of fresh semen was collected per sample per elephant and kept at -20°C/-80°C for further analysis.
Figure 2-5. (Large) Semen samples are collected using the manual stimulation technique. (Small) The protrusion of penis and the ejaculation are stimulated by massaging at ampulla area.

2.3 General EEHV diagnostic methods

Given that EEHV is yet to be cultured or isolated, confirmation of disease and infection is usually based on clinical pathology, molecular-based tests, and serological tests. The general EEHV diagnostic methods used in this study are described below. Specific details are described later in the relevant chapters.
2.3.1 *Clinical diagnosis*

Clinical diagnosis is an early diagnosis tool, based on the presence of clinical signs. Most EEHV haemorrhagic cases occur in young elephants (age range from 4 months to 18 years) (Zong et al., 2007), so it is important that this age class is routinely monitored for EEHV-related clinical signs. Early clinical signs of EEHV HD are non-specific, and include dullness, lethargy, and decreased appetite; all such clinical signs should be recorded and given further attention. Disease progression is indicated by clinical signs which often include swelling of the face and head (Fig. 2-6a), petechial haemorrhage at the tip of the tongue, and discoloration of mucous membrane (Fig. 2-6b) (Richman et al., 2000a; Richman & Hayward, 2012). Oral ulcers, abdominal discomfort, and lameness are also reported occasionally (Garner et al., 2009; Richman & Hayward, 2012).

**Figure 2-6.** Observed clinical signs during EEHV HD progression. (a) Swelling of the face and head due to subcutaneous oedema. (b) Petechial haemorrhage at the tip of the tongue, as the result of blood leakage from capillaries (Cracknell, 2008).
The progression of the disease is rapid and elephants usually die within 12–72 hours of clinical signs being first observed (van den Doel et al., 2015). However, as the disease begins with non-specific clinical signs, further diagnostic tests (i.e. cPCR, qPCR) are required to confirm clinical EEHV infection at an early stage, and whole blood samples should be collected to determine levels of viremia.

Adult elephants can also act as EEHV reservoirs, and carry the virus throughout their lives. If the virus reactivates in reservoir animals, non-pathogenic clinical signs may be observed, including ulceration and vesicle formation on oral mucosa and vaginal mucous membranes (Fig. 2-7). However, these are non-specific signs, and confirmation of the presence of EEHV from a lesion swab or biopsy requires further laboratory diagnostic tests.

**Figure 2-7.** Mucosal lesions (EEHV1-positive) which present in healthy adult Asian elephants. **(Left)** Oral ulceration (red arrow). **(Right)** Vesicular formation at the vestibular-vagina area (Schaftenaar et al., 2010).
2.3.2 Laboratory diagnosis

Haematology and biochemistry

Although haematology and biochemistry are not consistently altered during EEHV infection, specific trends in haematological and biochemical profiles are commonly detected in cases of EEHV infection, including leukopenia, thrombocytopenia, anaemia, and dehydration (Wiedner et al., 2012). At least 1 ml of whole blood is required to test for blood haematology, and at least 2 ml of serum is used for blood chemistry analysis. In the present study, blood values from known clinical EEHV cases were compared to standard blood values provided by the Species360 (Appendix 3), and any notable differences were recorded.

Conventional PCR

Conventional PCR, or standard PCR, is one of the most commonly used molecular-based diagnosis tests. This technique is used to detect pathogens by amplifying the DNA of interest, using repeated thermal cycles. Each thermal cycle of PCR consists of 3 steps: i) the *denaturing step*, where the two strands of DNA template (tested sample) are separated by heating up to 94°C–98°C; ii) the *annealing step*, where specific primers for the DNA of interest are bound to the complementary DNA strand at around 50°C–60°C; and iii) the *extension step*, where new complementary strands are produced using a thermostable polymerase enzyme and a mix of the four dNTPs at around 72°C. After completing the extension, two identical copies of the original DNA have been made. The same thermal cycle is then repeated, with the two new duplicated DNA strands being used as a template as well. Each duplicate creates two new copies; therefore after each cycle, the number of
copies of the original template is increased exponentially. The final product is detected by electrophoresis through a gel and staining with an intercalating dye such as ethidium bromide. The presence of a band of the expected size indicates a positive result.

Conventional PCR was the first method used to identify and confirm EEHV infection (Richman et al., 1999). In the present study, several EEHV-specific PCR primer pairs were chosen (as per previous research) to confirm EEHV infection and to obtain detailed genetic information by sequencing the PCR product (Zong et al., 2007; Garner et al., 2009; Stanton et al., 2010; Latimer et al., 2011; Zong et al., 2015). The nucleotide sequences of the primers used in this study are presented in Table 2-1. The total volume of each 50 µl PCR reaction comprised either: i) 45 µl of Platinum® PCR Supermix (Thermo Scientific, USA), 200 nM final concentration of each primer, nuclease-free water and DNA template; or ii) 25 µl of DreamTaq™ Green PCR Master Mix (Thermo Scientific, USA), 1 µM final concentration of each primer, nuclease-free water and DNA template. Further specific details, including the PCR conditions for each primer pair, are described in relevant later chapters.
Table 2-1. List of oligonucleotide primers used in this study.

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Quantitative real-time PCR

Quantitative real-time PCR is a technique developed from conventional PCR, which allows researchers to see and measure the accumulation of amplified product associated with the reaction. This sensitive and informative technique is less time consuming than conventional PCR, and does not require post-PCR manipulation. Real-time PCR techniques can be classified into two main groups: methods where the fluorescence of DNA-binding dyes is used to quantify product; and methods where fluorescent reporter probe is used.

This study used a method employing the DNA-binding dyes. The level of the PCR product amplified from the target DNA of interest is measured using one of the several fluorescent DNA intercalating dyes, which bind to all double-stranded DNA (dsDNA) present in the reaction. The increase in amplified product is monitored at each cycle by measuring the accumulating fluorescent signal. The amount of PCR product can be quantified, using either relative or absolute quantification methods. The relative quantification method measures the level of gene expression by comparing the signal from tested samples to reference samples, while an absolute quantification method measures the numbers of amplicons by comparing the fluorescent signal to a standard curve generated by amplification of serial dilutions of the same stretch of DNA. There are a number of DNA-binding dyes available, including SYBR® Green, EvaGreen®, LCGreen®, and SYTO® (Shipley, 2006). This study used SYBR® Green as the DNA binding dye, and used an absolute quantification method to quantify the viral copy number in the samples. Specific details, including the development of a SYBR Green I-based real-time PCR to screen for EEHV infection, are described in Chapter 4.
**Serological test**

Serological tests are used to measure specific antibody levels to antigens indicative the existence of disease state in body fluids, including blood serum. The novel serological tests for EEHV have recently been developed and reported (van den Doel et al., 2015). This developed technique uses viral envelope glycoprotein B (gB) of EEHV as an antigen to capture and measure antibody levels.

The gB-specific capture ELISA protocols used in this study followed those reported in van den Doel et al. (2015). Briefly, 100 ng/well of mouse-anti His antibody was used to coat in a 96-well microtiter plate. Then, 750 ng/well of purified His-tagged EEHV-gB antigen was bound to the capture mouse-anti His antibody. Then, 100 µl/well of the elephant serum, which had been diluted into two dilutions (1:100 and 1:200), was added. After incubation for 1 hour at 37°C, 100 µl/well of rabbit anti-elephant IgG was added, followed by the addition of swine anti-rabbit IgG HRPO conjugate (Fig. 2-8). Finally, the absorbance level, based on optical density (OD), was measured at 450 nm with 620 nm reference filter (A_{450/620}).

All available serum samples from each elephant were tested for antibodies against EEHV at 1:100 and 1:200 dilutions, and the absorbance (OD) ratio level was measured at each dilution by comparing with the background level (OD_{sample}/OD_{background}). Samples were considered ‘positive’ when both dilutions produced OD ratios three times greater than the background OD level (OD > 3), ‘undetectable’ when both dilutions produced OD ratios less than twice the background OD level (OD < 2), and ‘borderline/inconclusive’ when one or both of the dilutions produced OD ratios between two and three times the background OD level (OD = 2–3).
Figure 2-8. Components of the gB-specific capture ELISA used in this study for detecting EEHV1 infection.
CHAPTER 3

MOLECULAR CHARACTERISATION AND GENETIC VARIATION OF EEHV ISOLATED FROM CLINICAL CASES OF EEHV HD IN CAPTIVE YOUNG ASIAN ELEPHANTS IN THAILAND


I (Supaphen Sripiboon) undertook the field work, sample collection, sample processing, laboratory work on molecular diagnostics, initial write up and editing following review by supervisors. My supervisors (A/Prof Warren, Dr Jackson and Dr Holyoake) advised with regards to the study design for the research, provided epidemiological/statistical assistance and reviewed and edited the drafts of the manuscript. Professor Robertson provided additional epidemiological assistance for the study. Dr Ditcham provided supervision and training in molecular diagnostics. Dr Thitaram provided field work assistance. Dr Tankaew and Dr Lertwatchrasarakul provided in-country (Thailand) laboratory assistance.
3.1 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 and African elephants (Richman et al., 1999). EEHV haemorrhagic disease (EEHV HD) 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; Long et al., 2015).

The disease caused by EEHV occurs in both Asian and African elephants, but occurs most frequently in young Asian elephants, with an age range of affected individuals from four months to 18 years (Zong et al., 2007). Affected animals initially show non-specific clinical signs, such as lethargy and anorexia. Oedema 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 hours 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; Richman et al., 2000b). Death in these individuals is associated with organ failure or hypovolemic shock. The findings from necropsy examination consistently demonstrate extensive petechial to ecchymotic haemorrhage throughout the heart and most internal organs, with intranuclear 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 the 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; Long et al., 2015); therefore, molecular-based tests are primarily used for disease diagnosis. Seven types of EEHV have been identified (EEHV1–7), with differences in pathogenicity and host preference (Hayward, 2012). Among these, the most common type is EEHV1, which has two closely related subtypes: EEHV1A and EEHV1B (Richman & Hayward, 2012). EEHV1, EEHV3, EEHV4, and EEHV5 have been reported as the causative agents for EEHV-related mortalities of young 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; Fuery et al., 2016).

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., 2000a; Richman et al., 2000b). 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 & Thompson, 2001). However, given the increasing number of clinical EEHV cases reported in Asian elephants without a history of contact with African elephants, and no further evidence of EEHV in skin nodules of African elephants, this hypothesis has become more doubtful (Richman & Hayward, 2012; Zachariah et al., 2013; Long et al., 2015; Zong et al., 2015).
Despite the fact that EEHV has been studied intensively in captive elephants in 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 HD in Southeast Asia was reported (Reid et al., 2006). The affected animal was a three year old female wild-born Asian 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 et al. (2013) conducted a study of EEHV HD in South India; with nine out of 15 samples from dead young wild or orphaned 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 occasionally shed from adult Asian elephants which showed no clinical signs (Hardman et al., 2012; Stanton et al., 2010; Stanton et al., 2013), thus adult elephants could potentially play a role as a viral reservoir.

To obtain a better understanding of EEHV HD status in Asian elephant range countries, this molecular study tested suspected EEHV clinical cases, which had been submitted to EEHV diagnostic facilities in Thailand, to determine the viral genetic diversity and investigate the epidemiological relatedness of types, subtypes, and subtype clusters. The genetic characterisations of EEHV in Thai elephants were 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.

3.2 Materials and methods

3.2.1 Samples

A retrospective molecular study was conducted to re-examine samples of suspected EEHV clinical cases that were submitted to the four EEHV diagnostic facilities in Thailand between 2006 and 2014. These facilities were the Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen campus (FVM-KU-KPS); the Faculty of Veterinary Medicine, Chiang Mai University (FVM-CMU), the Faculty of Veterinary Science, Mahidol University (FVM-MU), and the Veterinary Research and Development Centre (lower north-eastern region) (VRD-LNE) (Fig. 3-1).

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 HD. General information including name, microchip 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 whilst still alive or immediately post-mortem. Only 12 cases were available for full post-mortem examination, and sample collection. The remaining cases did not have a full necropsy performed, generally only a tongue tissue sample was collected. 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.

Figure 3-1. Locations of all four EEHV diagnostic facilities in Thailand from which samples were obtained for this chapter (a) FVM-KU-KPS, (14.02081, 99.97322); (2) FVS-MU (13.79819, 100.31711); (3) FVM-CMU (18.7588, 98.94024); (4) VRD-LNE (14.77529, 103.43654).
3.2.2 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 protocols. 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 EEHV redundant polymerase primers (PANPOL) 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 samples. 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 samples 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 viral G protein-coupled receptor (vGPCR1/U51) genes. The details of primers used in this study are listed in Table 3-1, and amplification protocols followed were as previously described (Richman et al., 1999; Garner et al., 2009; Stanton et al., 2010; Latimer et al., 2011; Stanton et al., 2013; Zachariah et al., 2013).
Table 3-1. List of oligonucleotide primers used in this chapter.

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<tr>
<td></td>
<td>6752</td>
<td>-CTACATGCCCAGTAGCTAGG-</td>
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</tr>
<tr>
<td>vGPCR1</td>
<td>7506</td>
<td>-GATTGTGAACGCTGTATGTC-</td>
<td>910</td>
</tr>
<tr>
<td></td>
<td>4963</td>
<td>-GACTTTCTTCGTCGGCACTCGTCTT-</td>
<td>910</td>
</tr>
<tr>
<td></td>
<td>5200</td>
<td>-CGTGATACGTTCAACCATTACCA-</td>
<td>726</td>
</tr>
<tr>
<td></td>
<td>7470</td>
<td>-GGTGTTACGTGTATGATGTC-</td>
<td>747</td>
</tr>
<tr>
<td>TER3/4</td>
<td>A1</td>
<td>-GTGCTGTAGCAGGATCATGTC-</td>
<td>310</td>
</tr>
<tr>
<td></td>
<td>B1</td>
<td>-GTGCAACACGAGCAGCAAAG-</td>
<td>310</td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>-CGGATCATGTCGACTCCCGTG-</td>
<td>290</td>
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<td>Fwd</td>
<td>-CGTTGAAGGTGTTCGAGAT-</td>
<td>390</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>-CAGCATCATCCAGGCTACAAC-</td>
<td>390</td>
</tr>
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</table>
Briefly, the cycling conditions for PANPOL and TER primers were 94°C for 5 minutes for enzyme reactivation, followed by 35 cycles of 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute, then a final extension step of 72°C for 7 minutes. Other primers were used in PCR with the following conditions: 95°C for 2 minutes, then 45 cycles of 95°C for 40 seconds, 50°C for 45 seconds, and 73°C for 1 minute, then followed by 73°C for 5 minutes for final extension. The total volume of each 50 µl PCR reaction comprised either 45 µl of Platinum® PCR Supermix (Thermo Scientific, USA), 200 nM final concentration of each primer, DNA template, and nuclease-free water; or 25 µl of DreamTaq™ Green PCR Master Mix (Thermo Scientific, USA), 1 µM final concentration of each primer, DNA template, and nuclease-free water to volume. Sterile water was used instead of DNA template as a negative control to check for any contamination. The PCR product was electrophoresed through a 1.5% agarose gel, stained with GelRed™ Nucleic Acid Gel Stain (Biotium Inc., USA) and imaged using a Gel Doc™ EZ Imager (Bio-Rad Laboratories, USA).

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 (Altschul et al., 1990), 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. 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 vGPCR locus, 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 & Bryant, 2006). Nucleotide substitution of Kimura 2-parameter (K2P) model was used in the analysis and branch support was estimated by bootstrapping with 1,000 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.
3.3 Results

3.3.1 Clinical EEHV infection in Thailand

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 unwell elephants. The cases were distributed across the country of Thailand, and all elephants were classified as young elephants, aged less than 15 years (Arivazhagan & Sukumar, 2008). Most individuals were captive-born, and of the two exceptions, one was hand-raised in captivity from six months of age.

During a 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. 3-2), and all cases were from different captive elephant facilities. Details of EEHV-positive cases are described in Table 3-2.

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 EEHV infection. Lameness was observed in one case.
(ThE09), and bloody diarrhoea was also presented in one case (ThE18). Only 12 cases had a full post-mortem examination, and associated sample collection, undertaken at the time of death. The remaining cases did not have a full necropsy performed, generally only a tongue tissue sample was collected. The necropsy examination of those 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 wild baby elephant found dead in the forest, a newborn baby that had been attacked by its mother, a late-term stillborn elephant, and three baby elephants that died with an unclear history. None of the samples tested from these elephants was positive by molecular screening in this study.

**Figure 3-2.** Retrospective testing on samples collected from suspected EEHV clinical cases occurring between 2006 and 2014, presenting the total of 18 EEHV HD confirmed cases in Thailand. The EEHV HD-positive cases ranged across the country, and each red dot indicates each case.
Table 3-2. Details (sex, age, location, status) with laboratory result (PCR-based) of each EEHV HD-positive case found in this study.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>YOC 1</th>
<th>Type of sample</th>
<th>Sex</th>
<th>Age</th>
<th>Location in Thailand</th>
<th>Status</th>
<th>Outcome</th>
<th>Lab result</th>
<th>Subtype cluster 2</th>
<th>GenBank Accession No.</th>
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<tr>
<td>ThE01</td>
<td>2006</td>
<td>Heart</td>
<td>M</td>
<td>2yr</td>
<td>Central</td>
<td>Captive born</td>
<td>Dead</td>
<td>EEHV1A</td>
<td>A2</td>
<td>KT390749, KT390752, KT390755, KT390758, KT390761</td>
</tr>
<tr>
<td>ThE02</td>
<td>2008</td>
<td>Heart</td>
<td>M</td>
<td>2yr</td>
<td>South</td>
<td>Captive born</td>
<td>Dead</td>
<td>EEHV1A</td>
<td>D2</td>
<td>KT390747, KT390751, KT390753, KT390756, KT390760</td>
</tr>
<tr>
<td>ThE03</td>
<td>2008</td>
<td>Heart</td>
<td>M</td>
<td>2yr2mt</td>
<td>North-east</td>
<td>Captive born</td>
<td>Dead</td>
<td>EEHV1A</td>
<td>D2</td>
<td>KT390748, KT390750, KT390754, KT390757, KT390759</td>
</tr>
<tr>
<td>ThE04</td>
<td>2009</td>
<td>Heart</td>
<td>F</td>
<td>2yr</td>
<td>South</td>
<td>Captive born</td>
<td>Dead</td>
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<td>B3</td>
<td>KT447182-KT447186</td>
</tr>
<tr>
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<td>2011</td>
<td>Heart</td>
<td>F</td>
<td>5yr</td>
<td>East</td>
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<td>Dead</td>
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<td>A2</td>
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</tr>
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<td>2012</td>
<td>n/a 1</td>
<td>M</td>
<td>1yr2mt</td>
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<td>Dead</td>
<td>EEHV1A</td>
<td>D1</td>
<td>KT447192-KT447196</td>
</tr>
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<td>ThE07</td>
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<td>F</td>
<td>2yr5mt</td>
<td>North-east</td>
<td>Captive born</td>
<td>Dead</td>
<td>EEHV1A</td>
<td>D2</td>
<td>KT447197-KT447201</td>
</tr>
<tr>
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<td>F</td>
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<td>D1</td>
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<tr>
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<td>F</td>
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<td>B1</td>
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<td>F</td>
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<td>M</td>
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<td>Dead</td>
<td>EEHV1A</td>
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<tr>
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<td>Blood</td>
<td>M</td>
<td>2yr</td>
<td>South</td>
<td>Captive born</td>
<td>Survived</td>
<td>EEHV1A</td>
<td>A2</td>
<td>KT596940-KT596944</td>
</tr>
<tr>
<td>ThE14</td>
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<td>Tongue</td>
<td>F</td>
<td>1yr2mt</td>
<td>North</td>
<td>Captive born</td>
<td>Dead</td>
<td>EEHV1A</td>
<td>D1</td>
<td>KT596945-KT596949</td>
</tr>
<tr>
<td>ThE15</td>
<td>2014</td>
<td>Blood</td>
<td>M</td>
<td>3yr</td>
<td>North</td>
<td>Wild born</td>
<td>Survived</td>
<td>EEHV1A</td>
<td>E</td>
<td>KT596950-KT596954</td>
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<tr>
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<td>Heart</td>
<td>M</td>
<td>1yr4mt</td>
<td>North-east</td>
<td>Captive born</td>
<td>Dead</td>
<td>EEHV4</td>
<td>-</td>
<td>KT596955</td>
</tr>
<tr>
<td>ThE17</td>
<td>2014</td>
<td>Heart</td>
<td>F</td>
<td>3yr8mt</td>
<td>North</td>
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<td>Dead</td>
<td>EEHV4</td>
<td>-</td>
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<td>ThE18</td>
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<td>Heart</td>
<td>M</td>
<td>2yr11mt</td>
<td>North</td>
<td>Captive born</td>
<td>Dead</td>
<td>EEHV4</td>
<td>-</td>
<td>JN788931</td>
</tr>
</tbody>
</table>

1 Year of sample collection
2 Subtype cluster based on vGPCR1 (EEHV1-specific locus)
3 Unidentified types of tissue sample
3.3.2 Detailed genetic analysis

To determine the types of EEHV in each elephant, the polymerase/U38 locus of each sample was 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, sequences from this study clustered with the EEHV1A, EEHV1B, and EEHV4 reference sequences, creating clear clusters with high support values (Fig. 3-3). 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.

**Figure 3-3.** Based on the polymerase/U38 locus, a phylogenetic dendrogram using the maximum likelihood method (Kimura 2–parameter model) reveals three types of EEHV found in this study.
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 (gM1, TER1, HEL1), where samples can be classified into EEHV1A and EEHV1B subtypes (Fig. 3-4 and Fig. 3-5).

**Figure 3-4.** A phylogenetic dendrogram was constructed using the maximum likelihood method (the Kimura 2-parameter model), revealing the relationship of all EEHV1-positive samples in Thailand. Based on the gM1/U77 locus, 104 parsimony informative sites out of 296 positions were apparent in a multiple alignment, and the dendrogram indicates two out of three subtype clusters of gM1 locus were found in Thailand.
Figure 3-5. Phylogenetic dendrograms were constructed using the maximum likelihood method based on the Kimura 2-parameter model, showing the relationship of all EEHV1-positive samples in Thailand at (a) terminase locus and (b) helicase locus. Based on the TER1/U60 and HEL1/U77 loci, 74 and 183 parsimony informative sites out of 239 and 495 positions were apparent in a multiple alignment, respectively.
The most variable locus (vGPCR) revealed sufficient genetic variation to allow identification of several previously identified subtype clusters in this study’s 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). The number of cases in each subtype cluster was compared with a previous report (Zachariah et al., 2013), and the data presented in Fig. 3-6. Based on analysis of the vGPCR1 locus, NJ and ML phylogenetic trees 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. 3-7).

**Figure 3-6.** Based on vGPCR/U51 locus, five major subtype clusters can be derived from EEHV1. The number of cases in each subtype cluster obtaining from this study was compared to the results in previous reports from India, North America, and Europe.
**Figure 3-7.** The phylogenetic network (neighbour-net model) was constructed using SplitsTree4, shows the five different subtype clusters of EEHV1 found in this study. Based on the vGPCR/U51 locus, 221 parsimony informative sites out of 575 positions were apparent in a multiple alignment.

**Note:** (*) indicates reference sequences; ( _ ) indicates elephants that had a history of sharing the same facilities at least once in their lifetime.
Analysis of the five different loci of EEHV1 (POL1, TER1, HEL1, gM1, vGPCR1) showed that none of the isolates was 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. 3-8. However, the EEHV4 genomes were found to be well conserved, with only a 1% difference, based on polymerase gene analysis.

**Figure 3-8.** 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.
3.4 Discussion and conclusion

Elephant Endotheliotropic Herpesvirus HD is one of the most concerning issues for elephant conservation presently, as it has been responsible for nearly 60% of all deaths of young elephants in captivity in North America and Europe (Hayward, 2012). Only limited information on this disease has been reported from Asian countries, the home range of Asian elephants. The first fatality associated with EEHV infection in Asian elephant range countries was reported in Cambodia in 2006 (Reid et al., 2006). Eight fatalities and two EEHV clinical cases were later reported in captive Thai elephants (Sripiboon et al., 2013; Lertwatcharasarakul et al., 2015), and at least nine fatalities associated with EEHV infection were reported in wild Indian elephants (Zachariah et al., 2013). The first fatal EEHV case in Laos PDR was recently reported (Bouchard et al., 2014). So at least 21 EEHV clinical cases have been reported and confirmed in Asian elephant range countries.

The number of affected animals is relatively low when compared with the total number of Asian elephants, which is estimated to be around 40,000–50,000 (Choudhury et al., 2008). However, given the limitations of EEHV diagnostic facilities in some countries, and also the lack of public awareness of this disease, it is very likely that a number of young elephants have died without being diagnosed, and that the number of EEHV clinical cases in range countries is higher than recorded in these reports.

This study involved the genetic characterisation and phylogenetic analysis of the majority of EEHV clinical cases that occurred in Thailand between 2006 and 2014. In Thailand there is no central registry for recording EEHV infection and clinical cases. Data collection instead relies on pooling results from the four diagnostic facilities in Thailand. Samples tested in this study (n=24) were from EEHV diagnostic facilities, therefore it is possible there were other cases of clinical EEHV infection that had not been submitted.
for diagnosis. A total of 18 cases were confirmed positive for EEHV HD, with only two clinical cases of EEHV HD surviving the infection after intensive treatment with human antiviral 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 (Hayward, 2012; Richman & Hayward, 2012; Zachariah et al., 2013). This study was the first to undertake phylogenetic analysis and to provide an overview of most of the known and suspected cases of EEHV HD that have occurred in Thailand to 2014 inclusive. 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 HD had been recorded in Thailand. Positive cases were distributed across Thailand, and more cases were found in areas with high elephant populations.

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 & Hayward, 2012), although it noted the issue of detecting an effect with small sample sizes (O’Brien et al., 2009). The affected elephants included in this study were all classed as young elephants, and this age-bias is a limitation for detecting any age association with EEHV HD. However, given an age association has been suspected and reported elsewhere (Zong et al., 2007; Hayward, 2012), and that the majority of confirmed clinical EEHV cases were juvenile elephants in this study, routine monitoring for EEHV HD 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 was recommended. Further, routine surveillance of the herd should be conducted to determine
the viral status of individuals across all age classes. This will assist with detecting any carriers or actively shedding individuals, as well as provide much-needed clarification on the epidemiology of the disease in captivity – including the nature of subclinical reservoirs, latency, and shedding patterns; viral reactivation period following the detection; and the presentation of disease across different age classes. For these reasons, as well as others, it is important to train captive elephants from a young age to undergo sampling for diagnostic and medical purposes.

Previous studies have shown that EEHV HD also occurs in wild elephant populations in South India (Zachariah et al., 2013), however in this study, all of the EEHV-positive cases were captive-born elephants, with the 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 sample from a truly wild individual was from a baby elephant found dead in the forest, and the result was negative for EEHV. Given the limited sampling of wild elephants, this present study 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 for use in remote areas, as the areas inhabited by wild elephants are often difficult to access. These surveillance activities must be underpinned by the further development of sensitive and specific serological diagnostic tools for screening wild elephants that may not be actively shedding virus for PCR detection.

As EEHV can be detected in body fluids from asymptomatic elephants (Stanton et al., 2010; Latimer et al., 2011; Hardman et al., 2012; Long et al., 2015), confirmation that a fatality is associated with EEHV infection requires the identification of EEHV-related
pathological signs. In this study, clinical signs associated with EEHV infection, 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 also presented, supporting the observation that this type of virus shows less selective organ tropism than other types, and causes more severe pathological effects in the animal (Garner et al., 2009; Sripiboon et al., 2013). Post-mortem examinations revealed moderate to extensive haemorrhage in most internal organs, particularly the heart. 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. It is also recommend that in situations where full necropsy cannot be performed, tissue samples from at least the tongue 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.

The 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 HD confirmed cases that occurred in North America. The results revealed that none of the cases was 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). This suggested that the disease occurred sporadically, and was likely to be derived from different animals. 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 this study, various subtype clusters were also found, and none of the viral isolates was genetically identical. As the herpesvirus is a fragile organism, it can only survive outside the host for a short period of time. This contributes to the low chance of viral transmission via contamination of the housing facilities, or the environment. However, the virus is transmitted mainly via close contact, and the normal behaviour of elephants (i.e. touching, mussing), enables viral transmission from one to another, and keeps the virus circulating within a herd (MacLauchlan & Dubovi, 2011d). EEHV is also shed in elephant secretions including trunk and conjunctiva (Stanton et al., 2010; Hardman et al., 2012; Atkins et al., 2013; Stanton et al., 2013), with evidence this shedding pattern is intermittent (Hardman et al., 2012), and therefore it is likely there is ongoing exposure within a herd. Although none of the cases in this study was 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. 3-8). This suggests that a single locus analysis may not be sufficient to draw a conclusion concerning epidemiological relatedness, and multiple gene analysis is needed. The findings from this 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 have shown that 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 (A1, A2, A3), B (B1, B2, B3), C, D (D1, D2), and E, can be distinguished by analysis of the vGPCR1 gene sequence (Zong et al., 2007; Zachariah et al., 2013). This 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 clusters (Fig. 3-6). Results from this study 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 3-2). However, this finding was different from that reported in South 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, this study reported one case of cluster C (THE12), while no cases of cluster C had been reported in Europe and South India (Zachariah et al., 2013). Although there are no reports on any differences in pathogenicity between each subtype cluster, obtaining this information is still useful to determine the epidemiological relatedness of each case. The similarity of common subtype clusters found in Thailand and North America could be linked to the source of those imported Asian elephants living in North America, however, further data collection and analysis are needed to confirm this.

Only EEHV1 and EEHV4 were found in this study, which concurred with previous reports (Sripiboon et al., 2013; Lertwatcharasarakul et al., 2015). Surveillance for subclinical EEHV infection was recently conducted on healthy captive and semi-captive Asian elephants in South India, where results revealed that the most common 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 this study, where none of the fatal cases was due to EEHV5 infection. Given these findings, one hypothesis is 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 there is also the potential that infection with EEHV5 could perhaps be protective against infection with a more pathogenic type. It would be valuable for future studies to consider this, and to 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; Fuery et al., 2016). Based on the unambiguous chromatograms obtained by direct sequencing of 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 this study only refer to the dominant type that was more likely to be causing the disease in the elephant at the time of sampling. In addition,
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 their use in the future is recommended.

Asian elephants in their home range not only play an important role in the ecosystem; they are also involved in the life of the human populace, its culture, and tradition. Therefore, disease threats to Asian elephants are important to characterise not only for conservation purposes, but also to mitigate the impact they may have on national and cultural icons, traditional ways of life, and employment in human communities throughout the region. Elephant Endotheliotropic Herpesvirus infection represents a significant challenge for elephant conservation, as the disease has been responsible for at least 60% of all deaths in young captive Asian elephants in Western zoos over the past 20 years (Hayward, 2012). This study examined the largest number to date of confirmed clinical cases of EEHV infection in Southeast Asia, and determined the occurrence of EEHV HD in a range country of Asian elephants. Clarifying the genetic characterisation and epidemiology of this virus, and associated disease in captivity and the wild 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. Further, 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 predisposing 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.
CHAPTER 4

DEVELOPMENT OF A SYBR GREEN I-BASED REAL-TIME PCR ASSAY TO SCREEN ELEPHANTS FOR EEHV INFECTION
4.1 Introduction

As described earlier in this thesis, Elephant Endotheliotropic Herpesvirus (EEHV) can cause severe fatal haemorrhagic disease in both Asian and African elephants, with fatal cases more often reported in young Asian elephants (Latimer et al., 2011; Hayward, 2012; Richman & Hayward, 2012). The disease has a rapid progression, and elephants usually die from heart and internal organ failure within 12–72 hours after presentation of the first clinical signs (van den Doel et al., 2015). The disease is treatable using human antiviral drugs, however the success of treatment depends on early initiation (Wiedner et al., 2012).

In order to start the treatment at this early stage, early diagnosis is required; and to obtain the early diagnosis, a rapid and informative diagnostic test is needed. As EEHV cannot yet be cultured or isolated, currently both the diagnosis of EEHV and confirmation of the specific viral strain rely on molecular-based techniques (Latimer et al., 2011; Richman & Hayward, 2012). The molecular diagnostics employ nucleic acid-based tests to facilitate detection, diagnosis, sub-classification, prognosis, and monitoring of response to therapy (Patrinos & Ansorge, 2010). For EEHV diagnostics, conventional PCR and TaqMan® Probe real-time PCR are the molecular tools that are commonly used at present (Stanton et al., 2010; Latimer et al., 2011; Stanton et al., 2012), however each of these tests has limitations. Therefore, this chapter describes the reasons why development of a new method for EEHV detection is required, and illustrates the design and implementation of a suitable alternative method.

Conventional PCR (cPCR) was the first molecular-based test to be developed to identify EEHV infection (Richman et al., 1999). This method and its adaptations have been used as a standard method for EEHV diagnosis for almost two decades (Latimer et al., 2011). Despite its simplicity, disadvantages were found when using cPCR for EEHV diagnosis,
including: i) the assay is time-consuming, and takes at least 3–4 hours to get a result; ii) post-PCR manipulation such as gel electrophoresis is required, which is also time-consuming and increases the risk of laboratory contamination and consequent false positives; iii) the test has a low assay sensitivity, which requires second or third round running for samples that have low levels of virus, such as samples from asymptomatic elephants, or from early stage of infection; and iv) viral levels in the samples cannot be quantified by this method. These limitations of cPCR are major obstacles to the development of an effective EEHV monitoring plan, for which a rapid, sensitive, and informative test is required.

To resolve the aforementioned limitation of cPCR, a quantitative real-time PCR assay for EEHV detection was developed. The hydrolysis probe real-time PCR (TaqMan®) was first used to screen for EEHV infection in asymptomatic elephants (Stanton et al., 2010). Due to its high sensitivity (low limit of detection), this assay could detect viral shedding in healthy elephant populations for the first time, and indicated that EEHV can be shed intermittently at a low level from asymptomatic elephants, which was particularly useful for disease monitoring (Stanton et al., 2010; Hardman et al., 2012; Stanton et al., 2013; Stanton et al., 2014). This assay could also detect the virus from the early stages of the disease, and allow monitoring of the dynamics of viral replication in individual elephants (Stanton et al., 2013). Applying this assay in EEHV monitoring programmes has led to a decrease in the number of fatalities from EEHV infection in the past few years, by increasing the likelihood of disease detection in the early stages; thereby contributing to better outcomes from treatment plans (L. Howard, Houston Zoo (USA), personal communication, 2015).
Despite the advantages of the hydrolysis probe real-time PCR method, six separate reactions are required to screen for all types of EEHV (Stanton et al., 2010; Stanton et al., 2012). As the cost of probe and reagents are relatively high, thus the cost to screen for the presence of all types of EEHV is extremely high. Even if the assay could be multiplexed, problems may occur with preferential amplification (Haycock et al., 2015). In addition, specific reagents, machines and technicians with a high level of expertise are required.

This project aimed to reduce the issues of assay sensitivity and cost associated with EEHV screening, by developing a screening method that not only provides good analytical sensitivity, but is both informative and affordable. To this end, and as this chapter describes in detail, I developed a SYBR Green I-based real-time PCR, to screen for EEHV infection using universal redundant primers. As part of developing this method, I evaluated the assay’s analytical sensitivity, specificity, and reproducibility. I developed a standard curve by using 10-fold dilutions of a known plasmid concentration to quantify the virus numbers in the sample. I also designed and initially tested a method to discriminate the strain of EEHV responsible for an infection, by using melt curve analysis of amplified DNA from a small (62 base pair) region of the polymerase gene. Finally, I applied this newly developed assay to a real case study, with results described in this chapter below.
4.2 Materials and methods

4.2.1 Primer selection

Primers used for screening all types of EEHV were selected from a previously published article (Latimer et al., 2011), 6710-F: 5’-ACAAACACGCTGTCRGT RTCYCCRTA-3’ and 6711-R: 5’-GTATTTGATTYYGCNAGYYTGAYCC-3’. These are redundant primers designed to amplify a 500 base pair fragment from the gene encoding the EEHV polymerase, and are called the ‘PANPOL’ primer pair for the rest of the chapter.

4.2.2 DNA extraction

A set of plasmids containing EEHV sequences was constructed as follows: DNA was extracted from tissue samples collected during necropsy examination of juvenile Asian elephants in Thailand, which had been confirmed to be positive for EEHV1A, EEHV1B, or EEHV4, using the FavorPrep™ Viral Nucleic Acid Extraction Kit I (Favorgen Biotech Corp., Taiwan). The extracted DNA was subjected to cPCR, and the amplicons sequenced to confirm the types of EEHV infection, using the following primer pairs: PANPOL, TER1, POL1, TER3/4, and POL3/4. Details of primer sequences, PCR protocols, and sequencing methods are presented in Chapter 3, Section 3.2.2. Extracted DNA samples from each type of EEHV, which had been confirmed by sequencing, were then kept to use as positive controls and for preparing a standard plasmid as outlined below.
4.2.3 Preparation of standard plasmid

In order to prepare a standard plasmid, we selected the PCR product of an EEHV1A-positive sample, detected in the cPCR reaction using the PANPOL primer, and confirmed by sequencing. Five microliters of purified PCR product, obtained using the FavorPrep™ Gel/PCR Purification Kit (Favorgen Biotech Corp., Taiwan), was mixed with 1 µl of T&A plasmid cloning vector (Fig. 4-1), buffer, and ligation enzymes from the RBC T&A Cloning Kit (Real Biotech Corp., Taiwan), following the manufacturer’s protocols. The mixture was incubated overnight at 16°C to allow the DNA fragment to ligate into the plasmid.

Figure 4-1. Plasmid map of T&A vector used in this study (from: http://www.snapgene.com/resources).
After incubation, the recombinant plasmid was used to transform into competent bacterial cells using the heat shock method adapted from Froger & Hall (2007). Briefly, 10 µl of recombinant plasmid was mixed with 100 µl of just-thawed JM109 bacterial competent cells (Real Biotech Corp., Taiwan), and then the mixture was placed on ice for 30 minutes. The mixture was then heat shocked (42°C, water bath) for 90 seconds, followed by incubation on ice again for 2 minutes. The transformed bacteria were then added to 900 µl of lysogeny broth (LB) medium (without antibiotic), and incubated at 37°C for 1 hour, whilst being shaken continuously to allow expression of the antibiotic resistance gene used for colony selection in the next step. After incubation, the transformed bacteria were plated onto LB agar containing 100 µg/ml ampicillin (Amp). The plate was incubated at 37°C for 16 hours to allow the bacterial host cell to grow. After incubation, five transformed colonies were randomly picked to check for insertion of the gene of interest by PCR. A positive colony was chosen and streaked for single colonies on another LB-Amp plate. A single colony was then picked and placed into 10 ml of LB medium containing 100 µg/ml with ampicillin, and incubated overnight at 37°C, shaking at 200 rpm.

Plasmid was then extracted from 2 ml of the overnight bacterial culture’s medium using FavorPrep™ Plasmid Extraction Mini Kit (Favorgen Biotech Corp., Taiwan), following the manufacturer’s protocols. The presence of the plasmid was checked by running 3 µl of the extracted plasmid DNA on a gel, and the insertion of the EEHV DNA was verified by sequencing. Linearized plasmid DNA concentrations were quantified with a spectrophotometer, and copy numbers were calculated with the following formula:
\[
\text{Copies/\(\mu\)l} = \frac{(6.02 \times 10^{23} \text{ copies})X \text{ (Plasmid concentration)}}{(\text{No. of base})X (660 \text{ g/base})}
\]

4.2.4 Establishment a SYBR Green I-based real-time PCR

The SYBR Green I-based real-time PCR reaction was performed with a 20 \(\mu\)l total reaction, consisting of 10 \(\mu\)l of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad laboratories Inc., USA), a 300 nM final concentration of each primer, DNA template, and nuclease-free water. The qPCR conditions were as follows: enzyme reactivation at 98\(^\circ\)C for 3 minutes, followed by 40 cycles of 94\(^\circ\)C for 10 seconds, 60\(^\circ\)C for 30 seconds, and 72\(^\circ\)C for 30 seconds. A melt curve analysis was performed following the final amplification. The temperature was gradually increased from 65\(^\circ\)C–95\(^\circ\)C, at a rate of 0.5\(^\circ\)C every 5 seconds, with continuous fluorescence measurement during this step. DNA samples previously confirmed as EEHV1A, EEHV1B, and EEHV4 were used as positive controls; and nuclease-free water was used as a no template control (NTC). The melting temperatures of each positive control were recorded.

4.2.5 Sensitivity, specificity, repeatability, and reproducibility

To determine the analytical sensitivity or the detection limit of the assay, triplicate 10-fold serial dilutions of the standard plasmid (from \(10^8\) copies/\(\mu\)l to 1 copy/\(\mu\)l) were made by diluting in nuclease-free water, and one microliter of each standard dilution subjected to SYBR Green I-based real-time PCR using the PANPOL primer pair, with the cycling conditions as detailed above. The baseline threshold calculated by the program software was used in the assay. The threshold cycle (Ct) and melting temperature (T_m) of each
dilution were recorded. Average Ct for each standard dilution and standard deviation (SD) were then calculated.

The assay analytical specificity was determined using DNA samples extracted from the blood of healthy elephants and from the tissue samples of dead elephants, which had all been shown to be EEHV negative based on individual histories and following two rounds of semi-nested PCR using PANPOL primer (Latimer et al., 2011).

Repeatability was determined by performing qPCR using dilutions of the standard plasmid from $10^6$ copies/µl to $10^4$ copies/µl. Each dilution was repeated three times (intra-assay) and performed on three different occasions (inter-assay or reproducibility). The average and standard variation values for the Ct were determined.

In addition, the same serial dilutions to test for assay sensitivity (from $10^9$ copies/µl to 1 copy/µl) were used in conventional PCR, in order to compare the detection limits of two assays when using the same primer pair (PANPOL primers). This PCR was run using DreamTaq™ Green PCR Master Mix (Thermo Scientific, USA) under the following conditions: 94°C for 5 minutes, then 40 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute, followed by a final extension step at 72°C for 7 minutes. Final PCR products were electrophoresed through a 1.5% agarose gel, and products stained with GelRed™ Nucleic Acid Gel Stain (Biotium Inc., USA) and viewed under UV illumination in a GelDoc™ EZ Gel Documentation System (Bio-Rad laboratories Inc., USA).
4.2.6 Strain discrimination by melt curve analysis

The polymerase gene sequences of six types of EEHV were imported from NCBI [GenBank accession no. HM568523 (EEHV1A), HM568559 (EEHV1B), HM568558 (EEHV2), JN983092 (EEHV3), JN983096 (EEHV4), JN983108 (EEHV5), HM060765 (EEHV6)] and aligned using BioEdit® (Ibis Biosciences, USA). Within the sequence of the polymerase gene, a conserved region with minimal variation among all known types of EEHV was chosen as the amplification target for melt curve analysis. Primers were designed to specifically amplify 62 base pair region from EEHV1 and EEHV4, which are the EEHV types that are currently found in Thailand (Fig. 4-2). The nucleotide sequences of these two specific primers were as follows:

- **POL1-fwd:** 5’-CACAGGGACAGACAATG- 3’
- **POL1-rev:** 5’-CCAATCGTTAACATCGGTCA- 3’
- **POL4-fwd:** 5’-CGCAGGGGCGAACTG- 3’
- **POL4-rev:** 5’-CCAGTCGTTGAAGGCTGC- 3’

**Figure 4-2.** A multiple alignment of six reference sequences of each known EEHV polymerase/U38 loci are shown: black highlight indicates the 62 nucleotide base pair fragment to be amplified; the pink highlight shows the nucleotide sequence of the forward primer specific for EEHV3/4; the blue highlight shows the reverse primer specific for EEHV3/4.
DNA obtained from elephants known to be infected with either EEHV1 or EEHV4 (confirmed by sequencing) was subjected to qPCR amplification, using both new primer pairs. The SYBR Green I-based real-time PCR reaction was performed with a 20 µl total reaction, consisting of 10 µl of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-rad laboratories Inc., USA), a 300 nM final concentration of each primer, DNA template, and added nuclease-free water to volume. The qPCR condition was as follows: 94°C for 3 minutes, followed by 25 cycles of 94°C for 10 seconds, 60°C for 30 seconds, and 72°C for 20 seconds. Melt curve analysis was performed following the final amplification step. The reaction temperature was gradually increased from 65°C–95°C, at a rate of 0.5°C every 5 seconds, with continuous fluorescence measurement during this step. The T_m of each positive sample was recorded.

4.2.7 Applying the technique to disease monitoring and detection

Blood and swab samples were collected from a three year old wild-born male Asian elephant, which had developed clinical signs of EEHV infection, but had been treated with acyclovir and survived. The FavorPrep™ Viral Nucleic Acid Extraction Kit I (Favorgen Biotech Corp., Taiwan) was used to extract the DNA from 200 µl of both whole blood and swab solution samples. Details of sample collection and preparation are described in Chapter 2 (Section 2.2.2 and Section 2.2.3). All DNA samples, plasmid standards, and NTC were subjected to qPCR using the PANPOL primers following the conditions described above. The number of EEHV genomes was calculated from a standard curve and recorded. In addition, positive samples from the qPCR were subjected to cPCR, for determining the sequence of the different gene loci (POL1, TER1, gM1, HEL1, vGPCR1) as described in Chapter 3, Section 3.2.2. The sequencing data were
analysed and edited using the BioEdit®, and phylogenetic trees and networks were constructed using MEGA6.

As part of a separate research project into artificial insemination in Asian elephants, semen samples were collected from nine bull elephants using the manual collection method described in detail in Chapter 2, Section 2.2.5. To evaluate the possibility of disease transmission through semen, DNA was extracted from 200 µl of fresh semen using the FavorPrep™ Viral Nucleic Acid Extraction Kit (Favorgen Biotech Corp., Taiwan), following the manufacturer’s protocols. The DNA was subjected to qPCR using the PANPOL primers and conditions described above, and the presence or absence of virus was reported.

4.3 Results

4.3.1 Melting temperature and standard curve analysis

Following the amplification, a melt curve analysis was performed to determine the specific T_m of the product. The T_m for the product amplified from DNA from an elephant infected with EEHV1A using the PANPOL primers was found to be 84.5°C (Fig. 4-3).
Figure 4-3. First derivative of the melt curve of the product, amplified from DNA extracted from blood from an EEHV1A-infected elephant using PANPOL primers, giving $T_m$ at 84.5°C.

When the assay was applied to EEHV1B and EEHV4, different $T_m$ were observed (Fig. 4-4a), suggesting that this assay can detect and discriminate between at least three types of EEHV. However, less sensitivity (showing a high Ct value), was found on EEHV4 when compared with other types (Fig. 4-4b).
**Figure 4-4.** Using samples taken from elephants infected with one of the three different types of EEHV found in Thailand, (a) shows the differences in $T_m$ (83, 84, 84.5$^\circ$C) after running a melt curve analysis; (b) shows Ct value obtained from each EEHV strain.
A standard curve was generated from the Ct values obtained by qPCR amplification of the 10-fold serial dilution, from $10^8$ copies/µl to 1 copy/µl of the plasmid containing PANPOL EEHV1A (Fig. 4-5). A linear relationship between copy number and Ct value was observed, with a correlation coefficient value ($r^2$) of 0.998.

**Figure 4-5.** Negative linear regression of Ct values, obtained when 10-fold serial dilutions (from $10^8$ copies/µl to 1 copy/µl) of EEHV1A plasmid were amplified in the qPCR reaction using PANPOL primers, demonstrating a concentration-related response.

### 4.3.2 Assay sensitivity and specificity

The analytical sensitivity of the assay was assessed by amplification of 10-fold serial diluted DNA plasmid standard. Results showed that the limit of detection of this assay (with clear $T_m$) was 10 copies (Fig. 4-6a). However, a clear melt peak was only obtained with input DNA copy numbers greater than $10^2$ copies (Fig. 4-6b). Therefore, for this study, the limit of detection was 100 copies of the plasmid.
**Figure 4-6.** The assay analytical sensitivity test used 10-fold dilutions of the standard plasmid from $10^8$–10 copies/reaction, (a) shows that the limit of detection of this assay was 10 copies, when Ct was considered; (b) shows T_m obtained from each dilution, and shows that the limit of detection was 100 copies when both Ct and T_m were considered.
To test the assays analytical specificity, this study used samples of elephant DNA which had previously tested negative for EEHV (using two rounds of semi-nested PCR) as negative controls. These samples included three blood samples from healthy adult Asian elephants, two tissue samples from adult Asian elephants that had died from non-EEHV related causes, and three tissue samples from a young Asian elephant that had died from non-EEHV related causes. None of these DNA samples was positive for EEHV by qPCR.

The same series of plasmid dilutions were also subjected to conventional PCR using the PANPOL primer pair. The results showed that the limit of detection was $10^3$ copies. A gel image after electrophoresis is presented in Fig. 4-7.

**Figure 4-7.** The detection limit of conventional PCR was $10^3$ copies, when using the same primer pair; and five microliters of PCR product loaded in each lane.
4.3.3 Assay repeatability and reproducibility

The repeatability and reproducibility of the assay were determined by amplification of triplicate samples of three dilutions of standard plasmid (10⁶–10⁴ copies/µl) on three separate occasions. Results with average Ct and SD are presented in Table 4-1. All the standard variations were less than 0.250, showing the ability of this assay to discriminate between two-fold dilutions in more than 95% of cases.

Table 4-1. To determine the repeatability of the assay, triplicate samples from each dilution (10⁶–10⁴ copies/µl) were amplified, and the average threshold cycle (±SD) was determined. The reproducibility of the assay was measured by performing the assay (using the same dilutions) on three different occasions.

<table>
<thead>
<tr>
<th>Standard plasmid concentration</th>
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<th>Repeatability</th>
<th>Reproducibility</th>
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<tr>
<td></td>
<td></td>
<td>Avg Ct</td>
<td>SD</td>
</tr>
<tr>
<td>10⁴</td>
<td>3</td>
<td>17.07</td>
<td>0.12</td>
</tr>
<tr>
<td>10⁵</td>
<td>3</td>
<td>21.26</td>
<td>0.34</td>
</tr>
<tr>
<td>10⁶</td>
<td>3</td>
<td>26.46</td>
<td>0.44</td>
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</tbody>
</table>

4.3.4 Strain discrimination using two short primer pairs

The primers designed to amplify the variable region from EEHV1 and EEHV4 were specific for the targeted strain; nothing was amplified when the primer pair specific for EEHV1 was used on DNA obtained from an elephant infected with EEHV4, and vice versa. In addition, the qPCR products obtained from each strain had different melting
points. Melting temperature analysis showed that the product obtained from EEHV1 melted at 76.5°C, and that from EEHV4 melted at 82.0°C (Fig. 4-8).

**Figure 4-8.** First derivative melt peaks obtained from qPCR products amplified when POL1 primers (76.5°C) and POL4 primers (82°C) were used with EEHV1 and EEHV4-positive samples, respectively.

![Melt Peak](image)

4.3.5 Applying a SYBR Green I-based technique to disease monitoring and detection

A total of 13 blood samples and four swab samples were collected from a juvenile Asian elephant that showed clinical signs of EEHV infection, and had been treated with acyclovir. All swab samples and nine of the blood samples were collected during the period of treatment, while the other four blood samples were collected after the treatment was ceased. Results showed that virus numbers in blood ranged from $5.5 \times 10^3$–$5.9 \times 10^6$ VGCs/ml of blood. Following intravenous treatment with acyclovir, virus levels decreased steadily; with only low numbers of virus ($<10^3$ VGCs/ml) detectable in blood for up to two weeks post resolution of clinical disease. No virus was detectable a month
post-treatment, and no virus was detected in trunk and conjunctival swab samples during the treatment period. Further phylogenetic analysis of multiple gene loci was undertaken, including sequences of the polymerase (POL), terminase (TER1), glycoprotein M (gM1), helicase (HEL1), and viral G protein-coupled receptor (vGPCR1) loci. All loci were sequenced and a distance-based phylogenetic tree (NJ method) was generated, which revealed the virus to be EEHV1A (subtype cluster E) (Fig. 4-9a and 4-9b).

**Figure 4-9.** The distance-based phylogenetic tree generated from EEHV viral sequence from an elephant calf in this study (red box), compared with reference sequences. (a) Based on analysis of the POL/U38 locus sequence, the sequence from the calf was closely related to EEHV1A. (b) Based on analysis of the vGPCR/U51 locus sequence, the sequence from the calf was assigned to subtype cluster E.
To determine the possibility of EEHV transmission through semen, 11 semen samples were collected from nine Asian elephant bulls. All sampled elephants were adults, with ages ranging from 20–49 years. No EEHV-related clinical signs were reported, and no other EEHV tests had been previously conducted in this group of elephants. None of the semen samples was positive by qPCR using the PANPOL primers.

### 4.4 Discussion and conclusion

Fatalities associated with EEHV infection in young elephants have been reported all over the world. At least 18 cases of clinical EEHV infection have been confirmed in Thailand in the last decade (details in Chapter 3, Section 3.3.1). Currently, the two preferred diagnostic techniques for EEHV diagnosis are semi-nested PCR (Latimer et al., 2011) and TaqMan® Probe real-time PCR (Stanton et al., 2010; Stanton et al., 2012). However, there are major limitations with both of these methods; semi-nested PCR may be prohibitively time-consuming, and the probe method may be prohibitively expensive. These limitations may be particularly challenging for developing countries, such as Thailand and other range countries for Asian elephants.

As previously discussed, a fast, reliable, and affordable assay was required for EEHV diagnosis; and prior to this study such an assay was lacking. This chapter reports the development of a SYBR Green I-based real-time PCR. This method can both reduce overall assay duration and yield strain information while remaining affordable (5x less expensive than currently used molecular tests). The study selected one of the preferred primer pairs (PANPOL), which is currently used for EEHV screening and diagnosis in
most of the EEHV diagnostic facilities. This primer pair is normally used for conventional PCR (Latimer et al., 2011).

Despite the time and cost advantages of using SYBR® Green dye, this dye can bind to any dsDNA, presenting the risk of getting a fluorescence signal from other PCR products (such as primer dimer or non-specific product). Therefore, melt curve analysis is highly recommended after every amplification, in order to identify non-specific amplification products. This research also showed that when melt curve analysis was used, the \( T_m \) of the products obtained using the qPCR assay with the PANPOL primers was sufficiently different to allow discrimination between at least three types of EEHV; 1A, 1B, and 4. Despite the good strain discrimination obtained during this study, it noted that the amplicon in this study was potentially too long to obtain good discrimination between all strains with melt curve analysis; and to confirm that the discrimination between the three strains was reliable, a larger number of samples was needed.

Although DNA extracted from tissue samples from an EEHV4 fatality case was used as a positive control, a low sensitivity of EEHV4 detection was observed when using this assay, so a new short specific primer pair was developed for use in further steps. The short amplicon, amplified by the new primer pairs encompassing the polymerase binding region, melted at temperatures nearly five degrees apart, enabling very clear discrimination between the two most common strains of EEHV in Thailand; type 1 and 4. Use of this technique, after the initial screening qPCR using PANPOL primers to confirm the strain of EEHV infection, would allow treatment decisions to be made more rapidly than is possible using the present technique; which is particularly valuable as different types of EEHV may have different pathogenicity (Garner et al., 2009; Richman and Hayward, 2012; Long et al., 2015). Further, the preliminary results regarding the
different melting temperatures obtained from these two strains of EEHV clearly indicate the possibility of using melt curve analysis of the amplicons obtained from qPCR targeted to this region, to allow easy and cheap identification of any strains of EEHV. This would improve the efficacy of any screening programme, and enhance epidemiological research into the spread of the virus. Future work using synthesised DNA encoding this region, with sequences obtained from NCBI, would allow an assay to be set up to discriminate between the seven types, EEHV1 to EEHV7.

Melt curve analysis of qPCR products amplified using the PANPOL primer pair showed that this technique can discriminate between at least three strains of EEHV – 1A, 1B, and 4 – which have been previously reported in Thailand (Sripiboon et al., 2013; Lertwatcharasarakul et al., 2015). Although this primer pair has previously been used to detect the presence of all types of EEHV using conventional PCR (Latimer et al., 2011), evaluation of the sensitivity and specificity of assays using these primers to target specific EEHV types is still required.

This research found that this SYBR Green I-based real-time PCR has a low limit of detection, and can detect the presence of 100 copies of EEHV DNA per reaction. This represents a ten-fold increase in sensitivity over cPCR, using the same primer pair. Applying this assay to a real case study showed that qPCR is able to detect the presence of EEHV in both clinical and subclinical phases of the disease. Given these findings, it is proposed that this method could be of benefit for detection and quantification of EEHV infection both when monitoring disease progression in individual animals, and when undertaking surveillance of elephant populations. The rapidity with which results are obtained will facilitate treatment; allowing appropriate treatment at an earlier stage of this rapidly progressing disease, where early initiation of treatment is critical for increasing
the likelihood of treatment success. In addition, viral levels in blood and secretions can be measured using this method, making an important contribution to treatment decisions and effective monitoring. A low level of virus (10³–10⁴ VGCs/ml) may be found circulating in elephant blood, without the animal showing any clinical signs; and in such a case no treatment would be indicated (Zong et al., 2007; Stanton et al., 2010; Stanton et al., 2013). However, if an increase in viral levels is observed, close monitoring should be conducted. Viral levels in the early stages of clinical disease were found to be around 10⁴–10⁵ VGCs/ml, and treatment should start as soon as possible if viral levels exceed this (Stanton et al., 2013).

Studies of human herpesvirus (HHVs) have shown that most of the HHVs, including Human Herpes Simplex Virus, Cytomegalovirus, and Epstein-Barr virus, can be found in semen samples; and these viruses potentially contribute to poor semen quality in male humans (Kapranos et al., 2003; Chen et al., 2013). In addition, previous studies have reported that EEHV is shed and can be detected intermittently in vaginal swabs from asymptomatic female elephants (Hardman et al., 2012), demonstrating that EEHV can be shed in reproductive secretions. In the present study’s testing of semen samples, none of the samples tested were positive for EEHV. However, these findings need to be interpreted with consideration of the limited sample size (n = 11), and the lack of initial testing to identify the infection status of each sampled elephant. Therefore, further longitudinal studies and serological tests are required to determine whether virus is shed in semen, before being able to discount this potential route of transmission. Likewise, although HHVs shedding in semen is related to infertility issues in humans, further investigation is required to determine whether this also occurs in elephants.
In conclusion, this chapter describes the development and initial implementation of a SYBR Green I-based real-time PCR for EEHV detection which provides rapid, sensitive, specific, and reproducible results. This assay represents an excellent tool for EEHV detection in clinical and subclinical cases, with the capacity to improve the success of treatment and prevention plans. The adaptation of this assay for use with field samples is discussed in the next chapter.
CHAPTER 5

EEHV SURVEILLANCE IN CAPTIVE ASIAN ELEPHANTS IN THAILAND USING REAL-TIME PCR
5.1 Introduction

Elephant Endotheliotropic Herpesvirus (EEHV) is a recently recognised subgroup of herpesviruses that can lead to acute and often fatal haemorrhagic disease in young Asian elephants. The virus was first documented in 1999, however retrospective studies have reported evidence of EEHV causing fatal disease in young elephants since the 1980’s (Richman et al., 1999; Richman et al., 2000a; Richman et al., 200b). To date, seven types of EEHV (types 1–7) have been reported, with differences in host specificity and pathogenicity (Hayward, 2012; Richman & Hayward, 2012; Long et al., 2015). The most common type found to cause fatal haemorrhagic disease in young Asian elephants is EEHV1A (Hayward, 2012); however, other types have also been reported to cause fatalities, including EEHV1B, EEHV3, EEHV4, and EEHV5 (Garner et al., 2009; Latimer et al., 2011; Richman & Hayward, 2012; Denk et al., 2012).

Similar to other herpesviruses, EEHV can be found in apparently healthy elephants without evidence of clinical signs (Stanton et al., 2010; Latimer et al., 2011; Stanton et al., 2014; Zong et al., 2015). Clinical signs are more likely to be severe and have rapid progression when the virus infects young elephants, which generally lack immunity to the virus (Zong et al., 2007; Hayward, 2012; Long et al., 2015). Once infected with the systemic form of the disease, an individual often dies within 12–72 hours after clinical signs present (see details of clinical signs in Chapter 1, Section 1.3.7). Treatment is largely unsuccessful, unless implemented early in the course of the disease (Wiedner et al., 2012). Over the past 20 years, EEHV has been responsible for 60% of deaths in young captive Asian elephants in North America and Europe (Hayward, 2012). This disease is therefore of major concern for captive Asian elephants, and requires robust diagnostic
and management protocols, to ensure accurate diagnosis and early initiation of treatment to minimise ongoing fatalities.

Despite the fact that EEHV has been intensively studied and monitored in captive Asian elephant populations in Western countries, the status of the disease in Southeast Asia – the region encompassing the Asian elephant’s range countries – is less well described. Fatalities associated with EEHV infection have recently been reported in both wild and captive Asian elephant populations in several countries in Southeast Asia, including Cambodia (Reid et al., 2006), Thailand (Sripiboon et al., 2013; Lertwatcharasarakul et al., 2015), India (Zachariah et al., 2013), and Lao PDR (Bouchard et al., 2014). However, the infection status of healthy Asian elephant populations (or those with asymptomatic infection or subclinical infection) is poorly described (Stanton et al., 2014).

In line with other herpesviruses, EEHV may enter latency if a host does not initially develop clinical disease or survives clinical infection. Herpesviruses can also reactivate, resulting in viral shedding, if the host is immunocompromised (Grinde, 2013). Previous studies have reported intermittent shedding of EEHV from healthy elephants in ocular, respiratory, and reproductive secretions (Stanton et al., 2010; Hardman et al., 2012), demonstrating subclinical EEHV infection can occur in healthy Asian elephants. However, the predisposing factors for EEHV reactivation and shedding are still unclear (Stanton et al., 2010). Understanding the prevalence of infection in each herd is fundamental for decision-making around health management of captive individuals, and ultimately conservation management of free-ranging individuals. Accurate identification of healthy individuals that are shedding the virus in a herd is crucial to better understand the epidemiology of the disease, as well as to facilitate quarantine and management plans.
Prevalence of EEHV infection in healthy Asian elephants has been reported previously in the UK and South India. In the UK study, all elephants in the collection (n = 6) were found to be positive for EEHV1, based on a longitudinal study comprising collection of weekly swab and trunk wash samples, and monitoring for EEHV using the TaqMan® Probe real-time PCR over a three month period (Hardman et al., 2012). The percentage of EEHV shedding amongst individuals was found to vary weekly, ranging from 0–66%.

In the South India study, the prevalence of EEHV infection was reported to be 35% (16/46), based on a cross-sectional study conducted on healthy Asian elephants (Stanton et al., 2014). Those studies together demonstrated that subclinical EEHV infection occurs in captivity both inside and outside Asian elephant range countries; and also showed that subclinical EEHV infection can be detected from both longitudinal and cross-sectional study designs.

Thailand has the world’s fourth-largest remaining Asian elephant population (Choudhury et al., 2008), and has previously reported fatalities associated with EEHV infection in young captive Asian elephants (Sripiboon et al., 2013; Letwatcharasarakul et al., 2015). At least 18 clinical cases of EEHV have been confirmed between 2006 and 2014 (details in Chapter 3, Section 3.3.1). Based on contact history and multiple gene analysis, disease occurred sporadically with no epidemiological relatedness observed amongst cases. Despite the confirmed clinical cases of EEHV infection in Thailand, PCR surveillance of a healthy elephant population in Thailand (n = 31) found an EEHV prevalence of 0% (95%CI: 0–11%) (Hildebrandt et al., 2005). The findings from Hildebrandt et al. (2005)’s study questioned whether the virus is absent from healthy elephant populations in Thailand, or whether the observed prevalence was related to limitations of the study, such as sample size or analytical sensitivity of the diagnostic tests used. To investigate these
questions and to determine the status of subclinical EEHV infection in Thailand, this study used new screening tools with higher analytical sensitivity, and a lower limit of detection (as described in Chapter 4, Section 4.3.2). I conducted a cross-sectional study and collected blood, conjunctival swab, and trunk swab from Asian elephants located across Thailand’s large captive population. Variables including types of sample collected (blood, conjunctival swab, and trunk swab), sex, age-class structures, locations, contact history, and serological status associated with EEHV detection were recorded, evaluated and reported.

5.2 Materials and methods

5.2.1 Study sites, animals, and sample types

This present study was conducted between September 2013 and March 2015, with samples collected from elephants at 20 study sites (elephant facilities) located across five different regions of Thailand (Fig. 5-1). These study sites were selected based on the authorised veterinarian’s visiting plan, to ensure for receiving the permission to collect samples from the respective elephant owners. A convenience sampling method was used for the cross-sectional study, the practical criteria for selection included accessibility and ability to collaborate with government veterinarians, geographical proximity, availability at a given time, and willingness of organisations to participate. The first elephants to be sampled were the trained elephants, followed by any elephant that came to the sampling platform, in order of arrival. The study aimed to collect blood samples, conjunctival swab, and trunk swab from each individual. Additional data collected for each elephant included
house name, location, microchip number, sex, age, and contact history with previous EEHV cases.

5.2.2 Sample collection protocols

All sample collection procedures were approved by Murdoch University’s Animal Ethics Committee (Permit No. R2582/13). Samples were collected from elephants trained using standard training techniques (positive reinforcement-based) to facilitate sample collection. Animals were only sampled if they were compliant and had been suitably trained; any that demonstrated reluctance to participate or cooperate with a particular sampling protocol were excluded from sampling.

Blood samples (minimum 2 ml) were collected from the ear vein into an EDTA blood tube and kept at 4°C during transportation. Conjunctival and trunk swabs were collected as described previously (Hardman et al., 2012). A sterile cotton swab soaked in phosphate buffered saline (PBS) or sterile saline solutions, was used to swab the conjunctiva or the nostrils of the trunk. Care was taken during trunk swabbing to avoid any dirt within the nostril. If an elephant was to close their eyes during sample collection, swabbing inside the eyelid was permissible. The swab was then kept in a 1.5 ml microcentrifuge tube containing 250 µl PBS solutions, and stored at 4°C during transportation. The tube containing the swab was centrifuged at 6000 x g for 3 minutes at 4°C or room temperature. The swab was then discarded and the supernatant was used for the DNA extraction in later steps.
**Figure 5-1.** Map of Thailand, showing the location of study sites (elephant facilities) used for EEHV sampling from Asian elephants from September 2013–March 2015. The sampling sites in this study covered five different regions of Thailand; North (N), West (W), Central (C), North-east (NE), and South (S).

Blood samples (minimum 2 ml) were collected from the ear vein into an EDTA blood tube and kept at 4\(^\circ\)C during transportation. Conjunctival and trunk swabs were collected as described previously (Hardman et al., 2012). A sterile cotton swab soaked in phosphate buffered saline (PBS) or sterile saline solutions, was used to swab the conjunctiva or the nostrils of the trunk. Care was taken during trunk swabbing to avoid any dirt within the nostril. If an elephant closed their eyes during sample collection, swabbing inside the eyelid was considered sufficient. The swab was then kept in a 1.5 ml microcentrifuge tube.
containing 250 µl PBS solutions, and stored at 4°C during transportation. The tube containing the swab was centrifuged at 6000 x g for 3 minutes at 4°C or room temperature. The swab was then discarded and the supernatant was used for the DNA extraction in later steps.

5.2.3 DNA extraction protocols

All samples were submitted for DNA extraction as soon as possible, usually within 48 hours of collection. If samples could not be extracted within 48 hours, samples were stored at -20°C. DNA was extracted from 200 µl of whole blood or swab solution using the FavorPrep™ Viral Nucleic Acid Extraction Kit (Favorgen Biotech Co., Taiwan), following the manufacturer’s instructions. Extracted DNA was kept at -20°C or -80°C until further analysis.

5.2.4 Quantitative real-time PCR for EEHV detection

A SYBR Green I-based real-time PCR was developed to detect EEHV infection in this study (details in Chapter 4). The redundant PANPOL primers were used in the qPCR; 6710-F: 5’-ACA AAC ACG CTG TCR GTR TCY CCR TA-3’ and 6711-R: 5’-GTA TTT GAT TTY GCN AGY YTG TAY CC-3’ (Latimer et al., 2011). The SYBR Green I-based real-time PCR reaction was performed with a 20 µl total reaction, consisting of 10 µl of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories Inc., USA), a 300 nM final concentration of each primer, DNA template, and nuclease-free water. The qPCR condition was as follows: enzyme reactivation at 98°C for 3 minutes, then 40 cycles of 94°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. In order to confirm specific product amplification, a melt curve analysis was performed post
amplification. The reaction temperature was gradually increased from 65°C–95°C (0.5°C every 5 seconds), with continuous fluorescence measurement. All qPCR reactions were performed in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc., USA). Absolute quantification was performed using the standard curve method, in order to quantify the viral copy number. Positive samples were then confirmed by nucleotide sequence analysis, in which each positive sample was subjected to run over cPCR using the PANPOL primer pair (Latimer et al., 2011). The PCR product was visualised using gel electrophoresis, and submitted for direct sequencing after purification using Favorprep™ GEL/PCR Purification Kit (Favorgen Biotech Co., Taiwan). Tested samples were considered positive when: i) the threshold cycle (Ct) value was less than 40; ii) the melting temperature matched the expected temperature; and iii) a nucleotide sequence matched an EEHV sequence in the database. Any sample not meeting these criteria was considered negative.

Sequences from positive samples were edited using BioEdit® (Ibis Biosciences, USA), and compared to reference sequences in the database using Blastn (Altschul et al., 1990) to verify the identity of the EEHV isolate. Reference sequences of the polymerase locus of each known EEHV type (1–7) were obtained from NCBI, pooled with sequences obtained in this study, and aligned with MUSCLE in MEGA6 (Tamura et al., 2013) using standard settings. Neighbour-joining (NJ) and maximum likelihood (ML) phylogenetic trees were constructed to ascertain the type of EEHV virus in each elephant. Prior to generation of the ML tree, a best-fit substitution model was selected based on MEGA recommendations. The Kimura 2-parameter model (K2+G) was used in this study. A thousand bootstrap replicates were performed for each tree.
5.2.5 Serological analysis

As part of a collaborative study, this study conducted serological testing on one elephant herd (n = 23) using a test developed for antibodies raised in response to EEHV1A infection. A variable number of stored frozen serum samples were available from all 23 elephants, obtained between 2005 and 2015. In addition, all 23 elephants were sampled at one time point in 2014, to obtain a blood sample for both serological analysis and qPCR for EEHV, to compare these test types.

The EEHV1A-gB-specific capture ELISA was used to measure antibody response, following the previously published protocol by van den Doel et al. (2015). All serum samples were tested at 1:100 and 1:200 dilutions; and the optical density (OD) was measured at each dilution, and compared with the background level (OD_{sample}/OD_{background}). A sample was considered ‘positive’ when both dilutions of serum resulted in OD levels three times higher than the background OD level, ‘undetectable/negative’ when both dilutions produced OD levels less than two times of the background level, and ‘inconclusive/borderline’ when one or both of the dilutions produced an OD between two and three times higher than the background levels. For the purpose of this study, the case definition for a seropositive individual from a single serum sample was any elephant classed as either ‘positive’, or ‘inconclusive/borderline’. This status was used for the comparison of the qPCR result to the serological testing in 2014, and hereafter I refer to this as ‘point serostatus’.

As antibody titres may fluctuate through time, and given each elephant had more than one serum sample available for testing, this study further assigned the overall serostatus of each animal in this herd according to the following criteria suggested by Taweekpoke et al. (2016): ‘remain positive (RP)’ when greater or equal to 80% of all serum samples
were seropositive; ‘occasionally positive (OP)’ when the number of seropositive samples were less than 80%, but number of seropositives combined with inconclusive/borderline were greater or equal to 50% of all samples tested; ‘remain negative (RN)’ when greater or equal to 80% of all samples were seronegative/undetectable; and ‘occasionally negative (ON)’ when the number of seronegative/undetectable samples were greater than or equal to 50%, but less than 80% of all samples tested. For the purpose of this study, the case definition for a seropositive individual where multiple serum samples were tested was any elephant classed as either ‘occasionally positive’, or ‘remain positive’, and this present study calls this serostatus hereafter ‘overall serostatus’.

5.2.6 Statistical analysis

To determine any statistical associations between risk factors and EEHV infection, and associations between molecular and serological results, I calculated odds ratios and p-values using Epitools (Sergeant, 2015). The level of significance was set at \( p = 0.05 \), and chi-square tests used where all categories were >5, with Fisher’s exact two-tailed tests used where any one category was <5.

5.3 Results

5.3.1 Locations and samples obtained

Elephants in the study came from 20 different captive facilities, representing five regions of Thailand (Fig. 5-1). A total of 362 individual captive Asian elephants were sampled, comprising 267 females, 93 males, and two that had no recorded sex. The ages of elephants in this study ranged from two months to 80 years old, with the median age being
30 years old. However, when divided by age-class structure according to Arivazhagan and Sukumar (2008), five elephants were babies (newborn to one year old), 36 elephants were juveniles (1–5 years), 58 were sub-adults (5–15 years), and 263 were adults (> 15 years). Based on external appearance, behaviour, and medical records, all sampled elephants in this study were considered to be healthy elephants. In addition, no EEHV-related clinical signs (skin lesions, oral ulceration, face swelling, tongue cyanosis, or petechial haemorrhage at the tip of the tongue) were observed in any of the elephants during the study period.

Although the aim of this study was to collect blood, trunk swab, and conjunctival swab from each elephant, this was not possible for some individuals, due to refusal by the elephant at the time of sampling, or constraints from the owner of the elephant. Therefore of the total animals sampled: 82/362 individuals had blood samples only; 63/362 individuals had conjunctival and/or trunk swab samples only; 148/362 had blood and conjunctival swabs; 29/362 individuals had blood and trunk swabs; and 40/362 individuals had blood, conjunctival swabs, and trunk swabs tested (Table 5-1). In all, a total of 217 elephants had both blood and at least one swab sample collected. This resulted in a total of 621 samples (blood, conjunctival swabs, and trunk swabs) from the 362 individual elephants. All samples obtained for qPCR screening were collected at one point in time for that individual. Therefore the results for the molecular tests do not reflect serial or repeat samples (as compared to serological screening results).
Table 5-1. Sampling types and sample sizes from captive Asian elephants in Thailand collected during 2013–2015.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Number of individuals</th>
<th>Total number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood only</td>
<td>82</td>
<td>82</td>
</tr>
<tr>
<td>Swab samples only (conjunctiva, trunk, or both)</td>
<td>63</td>
<td>65</td>
</tr>
<tr>
<td>Blood and conjunctival swabs</td>
<td>148</td>
<td>296</td>
</tr>
<tr>
<td>Blood and trunk swabs</td>
<td>29</td>
<td>58</td>
</tr>
<tr>
<td>Blood, conjunctival swab, and trunk swab</td>
<td>40</td>
<td>120</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>362</strong></td>
<td><strong>621</strong></td>
</tr>
</tbody>
</table>

5.3.2 Prevalence of EEHV infection in healthy populations, and genetic analysis

This study found 5.5% (20/362, 95%CI: 3.4–8.4%) of all elephants sampled were positive for EEHV infection on either blood, trunk swab, or conjunctival swab, when using quantitative real-time PCR to detect EEHV DNA. Initial results based on melt curve analysis showed at least two types of EEHV were found (Fig 5-2).
Figure 5-2. Differences in melting temperatures observed when melt curve analysis was conducted, indicating at least two closely related types of virus, which melted at 84°C and 84.5°C.

To further identify the types and subtypes of EEHV in each positive sample, the polymerase gene locus (POL) amplified was sequenced, and all sequences compared against reference sequences in the database (NCBI) using Blastn, and aligned with reference sequences. The alignment contained 206 parsimony informative sites out of 465 positions. With both NJ and ML phylogenetic analysis, the sequences from this study clearly clustered with the EEHV1A and EEHV1B reference sequences, with high support values (Fig. 5-3). Details of each positive sample are listed in Table 5-2.
**Figure 5-3.** A phylogenetic dendrogram of the polymerase/U38 locus, using the maximum likelihood method, based on the Kimura 2-parameter model (K2+G), showed that 80% (16/20) of EEHV-positive cases were closely related to EEHV1A, and 20% were closely related to EEHV1B.
Table 5-2. Location, sex, age, and age class of 20 EEHV-positive Asian elephants, when cross-sectional EEHV surveillance using SYBR Green I-based real-time PCR was conducted in a healthy captive population in Thailand during 2013–2015 (n = 362).

<table>
<thead>
<tr>
<th>Elephant code</th>
<th>Location</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Age class</th>
<th>Threshold cycle (Ct)</th>
<th>Viral sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Blood</td>
<td>Conjunctiva</td>
</tr>
<tr>
<td>TECC17</td>
<td>North</td>
<td>F</td>
<td>40</td>
<td>Adult</td>
<td>35.0</td>
<td>Neg</td>
</tr>
<tr>
<td>TECC32</td>
<td>North</td>
<td>F</td>
<td>7</td>
<td>Subadult</td>
<td>Neg</td>
<td>38.3</td>
</tr>
<tr>
<td>TECC38</td>
<td>North</td>
<td>M</td>
<td>23</td>
<td>Adult</td>
<td>Neg</td>
<td>34.3</td>
</tr>
<tr>
<td>TECC46</td>
<td>North</td>
<td>F</td>
<td>2</td>
<td>Juvenile</td>
<td>Neg</td>
<td>38.5</td>
</tr>
<tr>
<td>MT02</td>
<td>North</td>
<td>M</td>
<td>16</td>
<td>Adult</td>
<td>36.4</td>
<td>-</td>
</tr>
<tr>
<td>MT04</td>
<td>North</td>
<td>F</td>
<td>2</td>
<td>Juvenile</td>
<td>-</td>
<td>35.8</td>
</tr>
<tr>
<td>MT20</td>
<td>North</td>
<td>F</td>
<td>60</td>
<td>Adult</td>
<td>38.3</td>
<td>Neg</td>
</tr>
<tr>
<td>MT31</td>
<td>North</td>
<td>M</td>
<td>40</td>
<td>Adult</td>
<td>-</td>
<td>37.3</td>
</tr>
<tr>
<td>PF15</td>
<td>North</td>
<td>F</td>
<td>2</td>
<td>Juvenile</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CCR07</td>
<td>West</td>
<td>F</td>
<td>50</td>
<td>Adult</td>
<td>36.3</td>
<td>Neg</td>
</tr>
<tr>
<td>CCR11</td>
<td>West</td>
<td>F</td>
<td>53</td>
<td>Adult</td>
<td>35.9</td>
<td>Neg</td>
</tr>
<tr>
<td>TWC22</td>
<td>West</td>
<td>F</td>
<td>5</td>
<td>Juvenile</td>
<td>-</td>
<td>37.9</td>
</tr>
<tr>
<td>TWC24</td>
<td>West</td>
<td>F</td>
<td>2</td>
<td>Juvenile</td>
<td>-</td>
<td>38.2</td>
</tr>
<tr>
<td>MS03</td>
<td>West</td>
<td>F</td>
<td>18</td>
<td>Adult</td>
<td>35.3</td>
<td>-</td>
</tr>
<tr>
<td>SN02</td>
<td>West</td>
<td>F</td>
<td>16</td>
<td>Adult</td>
<td>37.1</td>
<td>Neg</td>
</tr>
<tr>
<td>WCH10</td>
<td>West</td>
<td>F</td>
<td>34</td>
<td>Adult</td>
<td>36</td>
<td>Neg</td>
</tr>
<tr>
<td>PNG07</td>
<td>South</td>
<td>M</td>
<td>39</td>
<td>Adult</td>
<td>Neg</td>
<td>-</td>
</tr>
<tr>
<td>PNG13</td>
<td>South</td>
<td>M</td>
<td>5</td>
<td>Juvenile</td>
<td>Neg</td>
<td>-</td>
</tr>
<tr>
<td>ZSU28</td>
<td>North-east</td>
<td>F</td>
<td>31</td>
<td>Adult</td>
<td>Neg</td>
<td>-</td>
</tr>
<tr>
<td>ZSU45</td>
<td>North-east</td>
<td>F</td>
<td>10</td>
<td>Subadult</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: age class was assigned followed Arivazhagan and Sukumar’s (2008) criteria; Babies (newborn–one year old), Juveniles (1–5 years), Subadults (5–15 years), and Adults (> 15 years).
To determine the level of virus in each of the positive samples, I undertook absolute quantification using standard curve analysis. The threshold cycle of positive samples ranged from 34.3–38.5, with an average of 36.8 cycles. DNA was extracted from 200 µl of either whole blood or swab solution; and viral levels in this study ranged from 5.5x10^3–3.1x10^4 VGCS/ml of blood (or swab solution). The lowest viral level that was detected in this study was 5.5x10^3 VGCS/ml of swab solution, and the limit of detection of this assay was 10 copies number/reaction or 2.5x10^3 VGCS/ml. The average viral level in blood was 1.6x10^4±7.2x10^3 VGCS/ml of whole blood, while the average viral levels in conjunctival and trunk swabs were 1.4x10^4±9.2x10^3 and 1.5x10^4±6.6x10^3 VGCS/ml of swab solutions, respectively.

5.3.3 Variables associated with EEHV infection and detection using qPCR

Of the 20 individuals that tested positive by qPCR screening for EEHV, five were individuals with only swab samples obtained, and 15 were from individuals with both blood and swab samples obtained (Table 5-2). Within the latter group, 10/15 were positive on their swab sample, and 5/15 were positive on their blood sample. Only three positive individuals had blood, trunk swabs, and conjunctival swabs obtained. No individual tested positive across two or more sample types; for instance if an individual was positive on the blood sample, it was negative on any other swab sample.

This study found no significant association between sex and likelihood of EEHV infection (OR = 1.05; 95%CI: 0.37–2.97; p = 0.93). Results for EEHV qPCR-positive samples by age class are presented in Table 5-3. The analyses found that juvenile elephants (1–5 years) were significantly more likely to be positive for EEHV infection by qPCR, when compared to all other age classes combined (OR = 4.46; 95%CI: 1.60–12.45; p = 0.05).
Table 5-3. Age class, number of samples, samples types, and EEHV qPCR results for Asian elephants sampled at captive facilities in Thailand. Odds ratios and p-values are presented for EEHV detection in each age class, with the comparative factor being the adult age class.

<table>
<thead>
<tr>
<th>Age class</th>
<th>Total individuals sampled</th>
<th>Sample types (n)</th>
<th>EEHV qPCR- positive</th>
<th>Prevalence (95% CI)</th>
<th>OR (95% CI)</th>
<th>p-value (fisher’s exact)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baby</td>
<td>5</td>
<td>B(0), S(4), BS(1)</td>
<td>0</td>
<td>0%</td>
<td>1.8</td>
<td>(0.1–34.9)</td>
</tr>
<tr>
<td>Juvenile</td>
<td>36</td>
<td>B(1), S(14), BS(21)</td>
<td>6</td>
<td>16.7%</td>
<td>4.2</td>
<td>(1.5–11.9)</td>
</tr>
<tr>
<td>Subadult</td>
<td>58</td>
<td>B(13), S(9), BS(36)</td>
<td>2</td>
<td>3.4%</td>
<td>0.8</td>
<td>(0.2–3.4)</td>
</tr>
<tr>
<td>Adult</td>
<td>263</td>
<td>B(68), S(36), BS(159)</td>
<td>12</td>
<td>4.6%</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Total</td>
<td>362</td>
<td></td>
<td>20</td>
<td>5.5%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: B indicates blood sample was taken; S indicates swab sample was taken; BS indicates both blood and swab samples were taken.
Results for qPCR testing of EEHV by region are presented in Table 5-4. Statistical analysis found no association between qPCR detection and either elephant camps or regions of Thailand. Among the captive facilities, four were previously recorded as having an EEHV-positive herd; however, this study found no association between EEHV detection in healthy populations and history of EEHV exposures (OR = 1.44; 95%CI: 0.56–3.73; p = 0.45).

**Table 5-4.** Number of samples divided by the regions, and the results for qPCR EEHV testing of healthy Asian elephants in Thailand between 2013 and 2015. Odd ratios and p-values for regional associations are presented, with the comparative factor being the west.

<table>
<thead>
<tr>
<th>Region</th>
<th>qPCR result</th>
<th>OR (95% CI)</th>
<th>p-value (fisher’s exact)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POS</td>
<td>NEG</td>
<td></td>
</tr>
<tr>
<td>North (n=141)</td>
<td>9</td>
<td>132</td>
<td>1.0 (0.4–2.9)</td>
</tr>
<tr>
<td>South (n=36)</td>
<td>2</td>
<td>34</td>
<td>0.9 (0.2–4.5)</td>
</tr>
<tr>
<td>Central (n=2)</td>
<td>0</td>
<td>22</td>
<td>0.3 (0–0.1)</td>
</tr>
<tr>
<td>North-east (n=50)</td>
<td>2</td>
<td>48</td>
<td>0.6 (0.1–3.2)</td>
</tr>
<tr>
<td>West (n=113)</td>
<td>7</td>
<td>106</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>20</td>
<td>342</td>
<td></td>
</tr>
</tbody>
</table>
5.3.4 Serological testing

As noted above, this study conducted serological testing on one elephant herd (n = 23), using a test developed for antibodies raised in response to EEHV1A infection (see Section 5.2.5 for details). As more than one serum sample was available for each elephant and serological results can fluctuate over time, we followed the criteria developed by Tawepoke et al. (2016). Results showed’, only 9% (2/23) of elephants in this herd were observed to be ‘remain seropositive (RP)’, while 39% (9/23) were ‘occasionally seropositive (OP)’. In contrast, 22% (5/23) of individuals were found to be ‘remain seronegative (RN)’, and 30% (7/23) were ‘occasionally seronegative (ON)’. Therefore, according to the case definition for seropositive in ‘overall serostatus’ for this present study, which includes all RP and OP individuals, 48% (11/23) of elephants in this herd were classed as seropositive for EEHV infection. There was no association between sex and positivity of the overall serostatus (OR = 1.67; 95%CI: 0.31–9.01; p = 0.68); neither were juveniles more likely to be seropositive when compared to all other age classes combined (OR = 2.44, 95%CI: 0.19–31.53; p = 0.59).

Variations in serostatus within individual elephants over the sampling period complicated interpretation of results. To help address these difficulties, the analysis was restricted to those serum samples collected in May 2014 (when samples from this herd were also collected for qPCR). Serological results for this period showed that five elephants tested seropositive, seven tested inconclusive/borderline, and 11 elephants tested seronegative. When considering ‘inconclusive/borderline’ as positive according to this study ‘point serostatus’ case definition, 52% (12/23) of these elephants were seropositive. Results of overall serostatus and point serostatus in each individual are presented in Table 5-5.
Table 5-5. Name code, sex, and age class of elephants in a herd (n = 23) for which both serological and molecular tests were conducted. Sampling period and total serum number is provided for each individual. Serological results (overall and point serostatus) and qPCR results are presented.

<table>
<thead>
<tr>
<th>Name code</th>
<th>Sex</th>
<th>Age class</th>
<th>Sampling period</th>
<th>#Serum samples</th>
<th>Sero-status*</th>
<th>Serostatus Overall</th>
<th>Serostatus Point</th>
<th>qPCR result</th>
</tr>
</thead>
<tbody>
<tr>
<td>KD</td>
<td>F</td>
<td>adult</td>
<td>2005-2015</td>
<td>88</td>
<td>RP</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>KK</td>
<td>M</td>
<td>subadult</td>
<td>2010-2014</td>
<td>5</td>
<td>RN</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>KL</td>
<td>F</td>
<td>juvenile</td>
<td>2012-2014</td>
<td>6</td>
<td>RN</td>
<td>Neg</td>
<td>Pos (Inc)</td>
<td>EEHV1</td>
</tr>
<tr>
<td>JP</td>
<td>M</td>
<td>adult</td>
<td>2004-2014</td>
<td>35</td>
<td>ON</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>JJ</td>
<td>M</td>
<td>adult</td>
<td>2005-2014</td>
<td>13</td>
<td>OP</td>
<td>Pos</td>
<td>Pos (Inc)</td>
<td>EEHV1</td>
</tr>
<tr>
<td>NM</td>
<td>F</td>
<td>subadult</td>
<td>2010-2014</td>
<td>10</td>
<td>ON</td>
<td>Neg</td>
<td>Pos (Inc)</td>
<td>Neg</td>
</tr>
<tr>
<td>NO</td>
<td>F</td>
<td>subadult</td>
<td>2010-2014</td>
<td>12</td>
<td>ON</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>AI</td>
<td>M</td>
<td>subadult</td>
<td>2010-2014</td>
<td>10</td>
<td>OP</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>PJ</td>
<td>F</td>
<td>adult</td>
<td>2005-2014</td>
<td>35</td>
<td>OP</td>
<td>Pos</td>
<td>Pos (Inc)</td>
<td>Neg</td>
</tr>
<tr>
<td>PTD</td>
<td>F</td>
<td>adult</td>
<td>2005-2015</td>
<td>89</td>
<td>ON</td>
<td>Neg</td>
<td>Neg</td>
<td>EEHV1</td>
</tr>
<tr>
<td>PM</td>
<td>M</td>
<td>adult</td>
<td>2005-2014</td>
<td>34</td>
<td>RN</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>PP</td>
<td>F</td>
<td>adult</td>
<td>2005-2015</td>
<td>89</td>
<td>RP</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>MJ</td>
<td>F</td>
<td>adult</td>
<td>2010-2014</td>
<td>31</td>
<td>RN</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>LK</td>
<td>F</td>
<td>juvenile</td>
<td>2005-2015</td>
<td>35</td>
<td>OP</td>
<td>Pos</td>
<td>Pos (Inc)</td>
<td>Neg</td>
</tr>
<tr>
<td>WNL</td>
<td>F</td>
<td>adult</td>
<td>2005-2014</td>
<td>15</td>
<td>OP</td>
<td>Pos</td>
<td>Pos (Inc)</td>
<td>Neg</td>
</tr>
<tr>
<td>SK</td>
<td>F</td>
<td>adult</td>
<td>2005-2015</td>
<td>26</td>
<td>ON</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>ALN</td>
<td>F</td>
<td>subadult</td>
<td>2010-2014</td>
<td>5</td>
<td>OP</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>JO</td>
<td>M</td>
<td>subadult</td>
<td>2012-2014</td>
<td>8</td>
<td>OP</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>TW</td>
<td>M</td>
<td>adult</td>
<td>2011-2014</td>
<td>8</td>
<td>OP</td>
<td>Pos</td>
<td>Pos (Inc)</td>
<td>Neg</td>
</tr>
<tr>
<td>BL</td>
<td>M</td>
<td>juvenile</td>
<td>2012-2014</td>
<td>8</td>
<td>OP</td>
<td>Pos</td>
<td>Pos</td>
<td>EEHV1</td>
</tr>
<tr>
<td>BT</td>
<td>F</td>
<td>subadult</td>
<td>2010-2014</td>
<td>10</td>
<td>ON</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>PC</td>
<td>F</td>
<td>subadult</td>
<td>2012-2014</td>
<td>6</td>
<td>ON</td>
<td>Neg</td>
<td>Neg</td>
<td>EEHV1</td>
</tr>
<tr>
<td>PPH</td>
<td>M</td>
<td>subadult</td>
<td>2010-2014</td>
<td>5</td>
<td>RN</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
</tbody>
</table>

Note: EEHV-positive by qPCR (grey hi-light); (*) = serostatus based on Taweepoke et al.’s (2016) criteria.
Sampling for qPCR testing of the 23 individuals in this herd took place in May 2014. The ages of the elephants in this group ranged from 4–50 years, representing nine males and 14 females (Table 5-5). Molecular testing showed 22% (5/23) of elephants from this herd were positive for EEHV on qPCR (Table 5-5). Genetic analyses found that all positive samples from this herd were closely related to EEHV1A, with 80% (4/5) of positive results coming from swab samples.

When comparing point serostatus to qPCR results, and when ‘inconclusive/borderline’ was considered as seropositive, there was a lack of association between these two methods. Table 5-6 presents the data on the lack of association between point serostatus and qPCR results.

**Table 5-6.** For the 23 elephants tested in this study during May 2014, a lack of association was found between serostatus and qPCR detection.

<table>
<thead>
<tr>
<th>Serostatus</th>
<th>Molecular detection (qPCR)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td><strong>Seropositive</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Positive (distinct)</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>- Inconclusive/Borderline</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td><strong>Seronegative</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>5</td>
<td>18</td>
</tr>
</tbody>
</table>
5.4 Discussion and conclusion

Here I report findings from the first cross-sectional study to be undertaken across different regions and captive facilities in Thailand, to evaluate apparent EEHV prevalence in healthy Asian elephants using the qPCR method. The population of captive Asian elephants in Thailand is estimated to be around 3000–3500 individuals (Angkawanish et al., 2009); therefore, samples from this study represent approximately 10% of the total captive Asian elephant population in Thailand. The overall prevalence of subclinical EEHV infection in this study was 5.5% (20/362), of which all were positive for EEHV1. Results from this study were similar to the findings from previous research conducted in South India, where the prevalence of EEHV1 was reported to be 6.5% (95%CI: 1.4–17.9%) (Stanton et al., 2014). Based on melt curve analysis and nucleotide sequencing, the most common type of EEHV detected in this study was EEHV1A (16/20), which is the type most frequently responsible for EEHV-related fatalities in young elephants across the world, and in Thailand (Hayward, 2012; Richman & Hayward, 2012; Lertwatcharasarakul et al., 2015; Long et al., 2015).

The first attempt to investigate the status of EEHV infection in Thailand was conducted by Hildebrandt et al. (2005), using whole blood samples and biopsy tissue from retropharyngeal lymph nodes collected from 31 healthy captive Asian elephants in one herd. In that study, none of the samples was found to be positive for EEHV, based on the results from conventional PCR detection methods. Given the sample size, the upper limit of detection was 11.2%, which is greater than the 5.5% prevalence reported in this study. Further, this upper limit does not take into account the analytical sensitivity of the assay, which is negatively affected by intermittent shedding of the virus, as well as latency issues (Hildebrandt et al., 2005; Latimer et al., 2011). Due to low numbers of virus being shed,
the analytical sensitivity of conventional PCR appears insufficient to detect EEHV in healthy populations. Therefore nested PCR or qPCR should be used to screen elephants for subclinical EEHV infection (Stanton et al., 2010; Latimer et al., 2011), and testing should be undertaken on multiple samples collected from each individual over time to improve sensitivity. Despite the negative results from the EEHV screening conducted in 2001 (Hildebrandt et al., 2005), cases of fatal EEHV infection were reported in two captive Asian elephants in Thailand in 2013 (Sripiboon et al., 2013), indicating that the virus is present and active in captive elephants in Thailand.

Previous studies have shown that healthy elephants can shed EEHV via trunk, conjunctival, and vaginal secretions without developing any clinical signs (Hardman et al., 2009; Stanton et al., 2010; Latimer et al., 2011). Viral shedding is also intermittent; therefore, the likelihood of detection may vary on a weekly basis (Hardman et al., 2009; Stanton et al., 2010). The study from Hardman et al. (2009) found that in a herd of six captive Asian elephants, the weekly prevalence of EEHV detection ranged from 0 to 66%, and the overall prevalence over a three month period was 4.5%. However, a study by Stanton et al. (2010) reported an overall EEHV prevalence of 31% from samples collected from five captive Asian elephants, one to three times per week for approximately three months. These findings suggest that the observed prevalence of EEHV within a particular herd or area may be influenced in part by: i) sampling duration; ii) sampling frequency; iii) number of animals; iv) types of samples; and v) diagnostic tests used.

Although universal primers were used in the present study, no types of EEHV other than EEHV1 were found. In South India, another range country where Asian elephant research has been undertaken, the most common type of EEHV found in captive and semi-captive healthy elephants was EEHV5, at a prevalence of 20% (n = 46, 95%CI: 9.4–33.9%)
(Stanton et al., 2014). However, EEHV5 has not been reported in Thailand from healthy or clinically affected elephants, suggesting in combination with the findings from this study that EEHV5 may be either uncommon or absent from elephants in captivity in Thailand. However, this finding could also be due to a lower analytical sensitivity of this study’s test for EEHV5, as no EEHV5 positive control was available for the assay validation.

The levels of EEHV in blood (genome equivalents per millilitre) from clinical and non-clinical cases have been determined and described in previous research (Stanton et al., 2013). Stanton et al. (2013) found that low levels of virus (<10⁴ VGCs/ml) can be detected in the whole blood of asymptomatic elephants. Clinical signs were generally present when virus numbers exceeded 10⁴ VGCs/ml, and levels greater than 10⁶ VGCs/ml were observed in fatal cases (Stanton et al., 2013). The viral levels in this present study ranged from 10³–10⁴ VGCs/ml. No EEHV-related clinical signs were observed in positive cases. This concurs with the findings of Stanton et al. (2013); indicating that the diagnostic test described here is able to detect both subclinical and clinical infections (details of clinical cases are presented in Chapter 4, Section 4.3.5).

Although this study found that 75% (15/20) of EEHV-positive samples were from swab samples (either conjunctiva or trunk), only three positive individuals had blood, trunk swab, and conjunctival swab samples obtained, thereby limiting statistical analyses. The high variation in types of samples obtained from each animal would affect the analysis and interpretation of the results, therefore this study could not conclude if any sample type was more likely to detect EEHV infection in a healthy Asian elephant. To further investigate the likelihood of detection across different sample types, it is recommend future research focuses on parallel samples (blood, trunk swab, and conjunctival swab)
from a robust sample size, ideally as part of a longitudinal study. Based on the findings from this study, we suggest that for future investigations, sample sizes for parallel sampling issue should be at least 79 individual elephants (at population size of 3500, a confidence level of 0.05). In the meantime, as the results from this study found no association between viremia and viral shedding in trunk or conjunctival secretions, thus testing in parallel (e.g. multiple sample types from the same individual) is preferable to increase the chance of viral detection. As trunk wash samples have been reported to be a useful sample type for EEHV detection (Stanton et al., 2010; Latimer et al., 2011; Stanton et al., 2014; Bennett et al., 2015a), these should also be considered in future research evaluating preferred sample type for viral detection. Trunk wash samples were not collected in this study due to the limited number of trained elephants available for these procedures.

Previous studies have reported that the majority of EEHV viral shedding is found in adult elephants (Stanton et al., 2010; Hardman et al., 2012; Stanton et al., 2014). However, those studies had very small samples sizes in non-adult age classes; and furthermore they did not perform statistical analyses to determine if this association was significant. While this present study made efforts to convenience sampling in this study, there was an age bias as well as a selection towards those elephants that were trained and confident to present to the people collecting the samples (a behavioural bias). More than half of the samples in this study were from adult elephants; reflecting the fact that adult elephants are more likely to be conditioned to sampling, as well as the captive population having a higher proportion of adult elephants. It also found a bias towards females being sampled over males, which was expected given there is a sex bias towards females in captive facilities, owing to ease of handling (Lair, 1999; Thitaram et al., 2010). Despite the
skewed ratio towards adult elephants, this study found that EEHV was significantly more likely to be detected in juvenile elephants (1–5 years old) than all other age classes. This could suggest that the virus is more active in this age class; and it is recommended that regular monitoring (physical examination and routine blood tests) should be planned in order to prevent the clinical phase of EEHV infection in this age class particularly. No EEHV was detected in baby elephants (less than one year old), which could indicate the possibility of protective maternal immunity against EEHV infection. However, more samples from this age class and additional research is required to investigate this hypothesis.

There is limited peer-reviewed literature on the prevalence of subclinical EEHV infection in elephants, especially in Southeast Asia (Stanton et al., 2010; Hardman et al., 2012; Stanton et al., 2014; Bennett et al., 2015a), despite the recognition that subclinical infection and asymptomatic shedding of human herpesviruses are very important epidemiologically in humans (Mertz, 2008). In one cohort study, 4.7% of Herpes Simplex Virus (HSV) DNA was found in saliva samples taken from the oral cavities of a healthy human population (n = 1000) (Tateishi et al., 1994), whilst a longitudinal HSV study over a period of 1–2 months using oral swabs from healthy individuals found a prevalence of 76% (n = 30) (Knaup et al., 2000). These findings suggest that subclinical infection and asymptomatic shedding of HSV is common in humans, and that the observed prevalence can vary substantially in relation to the frequency and duration of the sampling regime.

Serological tests are useful tools to determine the immunological status of each animal within a herd, and for broader surveillance of infectious disease agents that are intermittently shed or enter latency in the host. The first serological test against EEHV1A has only recently been developed (van den Doel et al., 2015); and the present study was
able to apply this test in one particular herd where EEHV clinical cases had previously been reported (Boonprasert et al., 2015; Taweepoke et al., 2016). Multiple serum samples were available from each elephant in this herd for serological testing, and results showed considerable variation in serostatus within individuals during the sampling period, with only 9% and 22% of animals remaining either seropositive or seronegative respectively, throughout the period. This suggests that a rise and fall of antibody titres against EEHV infection may occur at different points through an elephant’s lifetime. When a specific narrow time period was considered, the present study found no association between molecular and serology results. This differs from a previous study by van den Doel et al. (2015), in which almost 80% correlation was reported. In most cases in the present study, seropositive elephants were qPCR negative. As viral shedding was intermittent, this study highlights the limitations of EEHV detection by qPCR when only one sample is tested. Further, the failure to detect the virus by qPCR in any particular elephant from this study did not indicate an absence of EEHV in that elephant, as the virus may not have been being shed at the same time that the sample was collected.

The aforementioned previous study by van den Doel et al. (2015) reported positive PCR results in six elephants which were seronegative (n = 36); and likewise, in the present study I recovered virus from two elephants which were seronegative. These two seronegative (but PCR positive) elephants from this present study did not have a historic serological response, and this negative serological result could possibly have been due to the early stages of infection, when IgM is more dominant than IgG – and this used ELISA test is based on the IgG response.

As one purpose of this present study was to compare the results between molecular and serological tests; and to facilitate interpretation, ‘inconclusive/borderline’ antibody titre
levels were counted as ‘seropositive’ for ‘point serostatus’. However, it should be noted that results may be interpreted differently if different criteria are applied; and the definition used here, which assumes that inconclusive/borderline titres are seropositive, could potentially lead to over-reporting of positive cases. Further research is required to investigate this possibility.

This is the first study to undertake EEHV surveillance in healthy captive Asian elephants across Thailand. This study found a prevalence of 5.5% in the population. However, this reported prevalence likely significantly underestimates the true prevalence of EEHV infection, due to the issues of shedding and latency associated with herpesviruses. Elephants that were positive for EEHV infection on qPCR, did not develop EEHV-related clinical signs. This suggests that subclinical EEHV infection (particularly EEHV1) occurs in the Asian elephant population in Thailand. Positive samples were taken from elephants across the country, and this study’s analyses revealed no association between location (or elephant facilities) and viral detection. In addition, positive samples were found in all age classes except the ‘baby’ class, with no association found between EEHV infection and sex. However, the virus was significantly more likely to be detected in juveniles. There was a lack of association between serological and molecular results. This suggests that more than one test, multiple samples, and serial sampling and testing are needed to increase the likelihood of viral detection and to rule out the presence of EEHV infection. This study also found that differences in sampling duration, frequency of sampling, and diagnostic methods (molecular compared to serology) impacted on EEHV detection. Further, no individual tested positive across two or more sample types, for instance if an individual was positive on the blood sample, it was negative on any other swab sample. These findings will be instrumental in formulating ongoing management programmes for
EEHV in Asian elephants. As the outcome of EEHV treatment relies largely on the timing of onset of treatment, knowledge of the EEHV status of individuals will facilitate timely treatment and increase the likelihood of positive treatment outcomes.
CHAPTER 6

LONGITUDINAL EVALUATION OF EEEV SHEDDING PATTERNS IN AN EEEV-POSITIVE ELEPHANT HERD IN THAILAND
6.1 Introduction

Elephant Endotheliotropic Herpesvirus (EEHV) infection is a disease of concern for elephant conservation, given the virus causes severe fatal haemorrhagic disease in young elephants (Hayward, 2012; Richman & Hayward, 2012). The virus and associated fatalities have been identified and documented since the late 1990s (Richman et al., 1999). EEHV1 was initially hypothesised to have evolved, via cross-species transmission, from a non-pathological infection in African elephants, to produce a sporadic and often fatal disease in Asian elephants (Richman et al., 1999; Zong et al., 2007; Richman & Hayward, 2012). This hypothesis was later rejected when several cases of the disease were reported in Asian countries, where no contact between the two species had occurred (Reid et al., 2006; Sripiboon et al., 2013; Zachariah et al., 2013; Bouchard et al., 2014). Clinical EEHV infection can be treated using human antiviral drugs, however treatment success is dependent on early intervention (Schmitt et al., 2000; Wiedner et al., 2012). At present, more than 80 cases of EEHV have been reported worldwide, with only 10 cases surviving (Hayward, 2012; Long et al., 2015). As treatment success is low and unreliable, and no vaccine for this disease is available; health and disease monitoring is paramount to disease diagnosis and management.

Molecular-based diagnostic tests have been standardly used for EEHV diagnosis and monitoring, as attempts to grow this virus have failed (Richman et al., 1999; Latimer et al., 2011). Quantitative real-time PCR assays have been developed and validated to detect most known types of EEHV (Stanton et al., 2010; Stanton et al., 2012). These assays have been used routinely for screening both healthy and clinically ill elephants in North America (Stanton et al., 2010). The assays are able to detect the virus before clinical signs occur, and are also able to detect the virus during shedding (Stanton et al., 2010; Stanton
et al., 2013; Stanton et al., 2014). Applying this technique for health and disease monitoring in a healthy population has shown that the virus can be shed intermittently from asymptomatic elephants; however, the frequency of viral shedding varies considerably in each individual (Stanton et al., 2010; Hardman et al., 2012), and the variables associated with EEHV shedding remain unclear.

Herpesviruses are structurally fragile and only survive for short periods of time outside their host’s body (Davison, 2010). Transmission typically requires close contact, particularly mucosal contact; such as during mating, licking, and nuzzling, especially between mother and offspring or between neonates (MacLauchlan & Dubovi, 2011d). It is presumed that such close contact would occur frequently in elephants, both captive and wild; and this could likely account for the circulation of EEHV within a herd (Ryan and Thompson, 2001). Indeed, when EEHV viral shedding was monitored in a previously clinically infected individual, it was clearly demonstrated that EEHV was circulating within the elephant’s herd, and that the frequency of virus detection varied amongst individuals (Stanton et al., 2010; Hardman et al., 2012; Atkins et al., 2013; Stanton et al., 2013). However, it is hypothesised that a captive herd (for example in a zoo, or in a managed facility in Thailand) could increase the spread of subtype variants due to inter-institutional transferring or contact between elephants originating from different localities (Ryan and Thompson, 2001).

Herpesviruses are successful colonisers, and through the establishment of latency, can avoid elimination by the immune system and have the ability to persist in a host for life after the primary infection (Grinde, 2013). During latency, the viral genome is retained, but the infectious virus cannot be detected. The latent virus can be reactivated to undergo renewed replication, often causing disease, in response to various stimuli (Preston, 2008).
However, given latency is controlled by the host’s immune system, the virus may also spontaneously reactivate; and may shed sporadically without the development of clinical signs (Wagner & Bloom, 1997). The exact stimuli that provoke reactivation are unclear, and at present the general term ‘stress’ is used (Preston, 2008). Research into latency continually reveals greater complexity in the interactions between a virus and its host, and the characteristics of latency also show variations when different herpesvirus subfamilies are studied (Grinde, 2013).

Latency is characterised by three phases: establishment, maintenance, and reactivation (Preston, 2008). During establishment, specific features of the natural host cell are required to prevent productive virus replication; for example, blocking immediate-early protein production – to inhibit virus replication, and minimise cytopathology and immune recognition (Preston, 2008). During maintenance, genomes are retained, and viral gene expression is minimised to hide the virus from the immune system. If repression of replication is not complete, low-level virus production may be blocked by the immune system. The reactivation phase involves the virus utilising cellular responses to a stimulus, but the stimulus may also damage the immune system and thereby cause disease (Preston, 2008). In humans, greater frequency of viral shedding and reactivation, leading to the development of clinical signs, is generally reported in patients who are immunocompromised (Sodroski et al., 1987; Bustamante & Wade, 1991; Wagner & Bloom, 1997; Grinde, 2013). As a consequence, it is hypothesised that immunosuppression could induce EEHV to reactivate and shed.

Immunosuppression can be related to disease, however stress and distress are also reported to impair immune function, leading to immunosuppression (Khansari et al., 1990). Stress can suppress both cellular and humoral immunity, by altering the
sympathetic pathway that influences the immune system (Segerstrom & Miller, 2004). Stress-induced herpesvirus reactivation has been reported both in humans and animals (Edington et al., 1985; Blondeau et al., 1993; Huang et al., 2011; Tanaka et al., 2012). Examples of stress-associated events in animals include concurrent infections, shipping, cold, crowding, and the administration of glucocorticoid drugs (MacLauchlan & Dubovi, 2011c).

To diagnose stress, the glucocorticoid hormone is commonly analysed (Möstl & Palme, 2002). Cortisol and corticosterone are the major forms of glucocorticoid hormone that increase following stress events/stressors via the hypothalamic-pituitary pathway (Möstl & Palme, 2002). Serum glucocorticoid concentration is a useful tool to assess animal welfare and monitor stress-related events, and has been applied in many species, including elephants (Schmid et al., 2001; Wilson et al., 2004; Fanson et al., 2013; Boyle et al., 2015). However, to analyse serum glucocorticoid concentration, blood collection is required; and this is an invasive technique that may in itself cause stress to the animal, which complicates interpretation. Therefore, non-invasive samples such as urine, faeces, and saliva are often preferable, and have been reported to provide satisfactory results (Wasser et al., 2000; Constable et al., 2006; Keay et al., 2006; Majchrzak et al., 2015). Although stress-induced herpesvirus reactivation may occur (Padgett et al., 1998; MacLauchlan & Dubovi, 2011c; Grinde, 2013), evidence of stress-induced EEHV reactivation and shedding is yet to be reported. A previous study reported a lack of correlation between EEHV shedding and cortisol levels (Bennett et al., 2015a); however, a small sample size meant the study’s power to detect a correlation would have been limited.
As part of this present research, I conducted a longitudinal study in Thailand, with the aim of monitoring EEHV shedding patterns in a captive Asian elephant herd containing an individual which had recovered from clinical EEHV infection. Given this individual’s close contact with other members of the herd, and the potential for herpesvirus transmission, this study compared shedding patterns and social relationships between elephants in the herd. Variables associated with diagnostic EEHV detection, including source of sample (for example blood or trunk swab), sampling frequency, and the duration of sampling were evaluated. The shedding pattern was compared to EEHV serological status, and the associations between the two diagnostic assays (molecular and serological) were analysed. Faecal samples were collected and glucocorticoid hormone testing were also undertaken, to investigate levels of stress occurring at that time. The concurrent occurrences of potentially stressful events were then recorded and evaluated. Potentially stressful events in elephants may include (but are not be limited to) concurrent infection or poor health status, changes in husbandry and management, extreme environmental change, translocation, and changes in herd status (Veasey, 2006; Carlstead et al., 2013). This study is unique in that it reports a longitudinal study of EEHV shedding patterns in an in-situ Asian elephant herd in Thailand, however logistical and management-related issues affecting sample size and frequency of sampling were apparent. More broadly, the novel findings from this study explore the clinical significance of EEHV in a herd containing an individual which has recovered from clinical EEHV infection; to inform the management of infected individuals and their conspecifics within a herd.
6.2 Materials and methods

6.2.1 Animals

The study was conducted from September 2014 to April 2015. Five captive Asian elephants at the Thai Elephant Conservation Center (National Elephant Institute, Forest Industry Organization, Lampang, Thailand; 18.250683, 99.2220751) were selected and monitored for EEHV infection, as they had all previously had contact with a clinical EEHV-positive elephant. These five Asian elephants included Elephant 1, a three year old orphan male wild-born elephant which had recently survived a clinical EEHV infection (in February 2014); Elephant 2, an approximately 30 year old female elephant which was the surrogate mother of Elephant 1 from six months of age to weaning; Elephant 3, a five year old female elephant, calf of Elephant 2, and which also lived with Elephant 1 before weaning; Elephant 4, a two year old female elephant that lived with Elephant 2 after Elephant 1 was weaned; and Elephant 5, an approximately 22 year old female elephant with a history of abortion. The small sample size was dictated by access to animals in collaboration with the government veterinarian and monetary budget. The relationship between each elephant is presented in Fig. 6-1. All elephants were physically examined and considered to be healthy during the sampling period.
Figure 6-1. Timeline showing the relationships between the elephants included in this study. *Elephant 5* is not included in this diagram as it lived in another sub-herd; however, this animal had contacted with *Elephant 2* on a regular basis.

6.2.2 Sample collection

All sample collection procedures were approved by Murdoch University’s Animal Ethics Committee (Permit No. R2582/13). Samples were collected from elephants trained with positive reinforcement-based standard training techniques. Animals included in this study were well-trained, however if an elephant demonstrated a reluctance to participate or cooperate in sample collection at a particular point in time, that elephant was excluded from the sampling regime.
We aimed to collect bi-weekly blood and swab samples, however, blood samples could only be collected monthly given access and permission was required from the elephant’s owners. Blood samples were collected monthly from each individual, except Elephant 4 as this elephant was yet to be trained for blood collection. A minimum of two millilitres of blood was collected from the auricular ear vein and placed into an EDTA coated tube. Samples were kept at 4°C during transportation, and DNA was extracted within 48 hours. If this was not possible, the samples were stored at -20°C and processed later.

Conjunctival and/or trunk swab samples were collected every two weeks. Sterile cotton swabs soaked in phosphate buffered saline (PBS) or sterile saline were used to swab the trunk or conjunctiva area of the elephant. Care was taken during trunk swabbing to avoid any dirt within the nostril. The swab was then kept in a 1.5 ml microcentrifuge tube containing 250 µl PBS solution. Samples were stored at 4°C during transportation, and centrifuged at 6000 x g for 3 minutes at 4°C or room temperature. The swab was then discarded, and the supernatant was used for DNA extraction in the next step. If DNA could not be extracted within 48 hours, the supernatant was then kept at -20°C, until further processing.

In addition, faecal samples were collected every two weeks, to evaluate concentration of glucocorticoid hormone and relate this to the occurrence of any potentially stressful events. Faeces were collected at the same time of day (early morning collection), to avoid diurnal variations in stress hormones. Approximately 10 grams of well-mixed fresh faeces were collected into a clean plastic bag. Samples were kept at 4°C during transport and stored again at -20°C, prior to further processing.
6.2.3 A SYBR Green I-based real-time PCR for EEHV detection

i) DNA extraction protocols

Blood and swab samples were submitted for DNA extraction, usually within 48 hours. If samples could not be extracted within 48 hours, samples were stored at -20°C. The DNA was extracted from 200 µl of whole blood or swab solution using FavorPrep™ Viral Nucleic Acid Extraction Kit (Favorgen Biotech Corp., Taiwan), following the manufacturer’s instructions. Extracted DNA was kept at either -20°C or -80°C until further analysis.

ii) Quantitative real-time PCR for EEHV detection

A SYBR Green I-based real-time PCR was developed to detect EEHV infection in this study (details in Chapter 4, Section 4.2.4). The redundant PANPOL primers were used in the reaction; 6710-F: 5’-ACA AAC ACG CTG TCR GTR TCY CCR TA-3’ and 6711-R: 5’-GTA TTT GAT TTY GCN AGY YTG TAY CC-3’ (Latimer et al., 2011). The SYBR Green I-based real-time PCR reaction was performed with a 20 µl total reaction, consisting of 10 µl of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories Inc., USA), a 300 nM final concentration of each primer, DNA template and nuclease-free water. The qPCR condition was as follows: enzyme reactivation at 98°C for 3 minutes, then 40 cycles of 94°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. In order to confirm the specific product amplification, a melt curve analysis was performed after amplification. In this step, the reaction temperature was gradually increased from 65°C–95°C (0.5°C every 5 seconds), with continuous fluorescence measurement. Absolute quantification using a standard curve method was performed to
quantify the viral copy number in each reaction. All qPCR reactions were performed using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc., USA).

To confirm the results using nucleotide sequence analysis, each positive sample was subjected to run over cPCR using the same primer pair (the PANPOL primers) (Latimer et al., 2011). The PCR product was visualised with gel electrophoresis, and submitted for direct sequencing after purification using the Favorprep™ GEL/PCR Purification Kit (Favorgen Biotech Corp., Taiwan). Nucleotide sequencing was undertaken 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 (Altschul et al., 1990), to verify the identity of the EEHV isolate. Tested samples were considered positive when i) the threshold cycle (Ct) value was less than 40; ii) the melting temperature (T_m) matched the expected temperature; and iii) a nucleotide sequence matching an EEHV sequence in the database was obtained. Any sample not meeting these criteria was considered negative.

Sequences from positive samples in this study were pooled with reference sequences of polymerase loci of each known EEHV types (1–7), and aligned with MUSCLE in MEGA6 (Tamura et al., 2013) using the standard setting. Neighbour-joining (NJ) and maximum likelihood (ML) phylogenetic trees were constructed to ascertain the type of virus present in each elephant. Prior to generation of the ML tree, a best-fit substitution model was selected based on MEGA recommendation. The Kimura 2-parameter model (K2+I) was used in this study. A thousand bootstrap replicates were performed for each tree.
6.2.4 EEHV1A-gB-specific capture ELISA

Stored serum samples were available for retrospective analysis for all elephants, except Elephant 4. EEHV1A-gB-specific capture ELISA was undertaken, following previously published protocols (van den Doel et al., 2015). All available serum samples from each elephant were tested for antibodies against EEHV infection in 1:100 and 1:200 dilutions, and the optical density (OD) level was measured in each dilution by comparing with the background level (OD_{sample}/OD_{background}). Samples were considered ‘positive’ when both dilutions produced OD ratios three times greater than the background OD level (OD > 3), ‘undetectable/negative’ when both dilutions produced OD ratios less than two times the background OD level (OD < 2), and ‘borderline/inconclusive’ when one or both of the dilutions produced OD ratios between two and three times the background OD level (OD = 2–3). Serostatus (based on OD value) in each elephant was then evaluated and reported.

6.2.5 Faecal corticosterone metabolite analysis

Faecal hormone was extracted using the ethanol boiling method (Mateo & Cavigelli, 2005). Prior to this, approximately five grams of faecal sample was incubated at 60°C for 24–48 hours until dry. The sample was then left at room temperature to cool and the grass content removed. Two grams of remaining faecal matter was then measured for hormonal extraction. Five millilitres of 90% ethanol was added in the glass tube containing dry faecal matter, and the tube was boiled in a water bath (96°C) for 20 minutes. Ethanol was then added to bring the volume up to approximately pre-boil levels, and the tube was centrifuged at 2000 x g for 20 minutes. The supernatant was extracted and placed into separate tubes, and ethanol was added to the remaining pellets and centrifuged again for 20 minutes. Supernatant from the first and second centrifuge runs were combined and
dried under air in a warm water bath. Three millilitres of 100% ethanol were added and sonicated for 15 minutes. Prior to analysis, the extracted hormones were reconstituted with methanol.

The concentration of faecal corticosterone metabolite was analysed using the enzyme immunoassay described by Brown et al. (2005). Prepared faecal samples were diluted 1:20 prior to analysis. Samples were assayed in duplicate. The analytical sensitivity of the assay was 0.1 ng/ml, and the inter-assay coefficient of variation (CV) for the high and low concentration controls were 5.62 and 4.00 %, respectively. The intra-assay CV for high and low concentrations were 2.63 and 1.26%, respectively.

6.2.6 Statistical analysis

To determine any statistical associations between risk factors and EEHV infection, and any association between molecular and serological results, odds ratios and p-values were calculated using Epitools (Sergeant, 2015). The level of significance was set at $p = 0.05$, and chi-square tests were used where all categories were $>5$, with Fisher’s exact two-tailed tests used where any one category was $<5$. 
6.3 Results

6.3.1 Variations in EEHV detection

Blood and swab samples were collected and tested for the presence of EEHV using SYBR Green I-based real-time PCR. During the study period, three of the five animals tested positive for EEHV on qPCR. The variation in frequency of viral detection in each elephant ranged from 0–19% and is reported below (Table 6-1).

Table 6-1. The frequency of detection of EEHV DNA in blood, conjunctival swab, and trunk swab samples obtained from five elephants. Each positive result was obtained at a different point in time in each elephant. The highest frequency of EEHV DNA detection was found in Elephant 1, which had survived clinical EEHV infection six months prior to this study.

<table>
<thead>
<tr>
<th>Elephant no.</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>No. of samples</th>
<th>No. of positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>4</td>
<td>16</td>
<td>3 (19%)</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>30</td>
<td>17</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>5</td>
<td>17</td>
<td>2 (12%)</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>2</td>
<td>12</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>22</td>
<td>20</td>
<td>1 (5%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td><strong>82</strong></td>
<td><strong>6 (7.3%)</strong></td>
</tr>
</tbody>
</table>
This study collected 82 samples in total, of which six samples (7.3%) tested positive for EEHV. Of these positive samples, 67% (4/6) were from blood, and 33% (2/6) from trunk swab samples. The univariant analysis revealed no significant difference in detection of EEHV from blood or swab samples (OR = 4.61; 95%CI: 0.79–26.96; p = 0.067). However, a univariant analysis revealed a significant difference between detecting EEHV in *Elephant 1*, which had survived clinical EEHV infection six months prior to this study, when compared to other elephants in the study (OR = 4.85; 95%CI: 0.88–26.74; p = 0.05).

Although viremia and EEHV shedding were detected, no clinical signs of EEHV disease were observed during this study period. Large variations in viral levels were observed, and viral levels during positive episodes ranged from $5.9 \times 10^3$–$1.7 \times 10^5$ VGCs/ml of blood or swab solution. The average viral level number was $4.5 \times 10^4 \pm 6.9 \times 10^3$ VGCs/ml.

Melt curve analysis revealed at least two different types of EEHV in this study, as evidenced by the differences in melting temperatures (83°C and 84.5°C) (Fig. 6-2). Nucleotide sequences confirmed two types of EEHV; EEHV1A (giving $T_m$ at 84.5°C) was found in five of six samples, and EEHV4 (giving $T_m$ at 83°C) was found in one sample from *Elephant 3*. 
Figure 6-2. Melt curve analysis of two positive samples: from Elephant 3 (yellow line) which gave a T_m at 83°C; and Elephant 5 (blue line) which gave a T_m at 84.5°C.

A phylogenetic tree was generated from pooled sequences of samples and references. Based on the polymerase locus, multiple sequences alignment was conducted, which revealed 211 parsimony informative sites out of 455 positions. For both NJ and ML phylogenetic analysis, sequences from this study clustered with the EEHV1A and EEHV4 reference sequences, creating clear clusters with high support values (Fig. 6-3).
Figure 6-3. A phylogenetic dendrogram was constructed using the maximum likelihood method (Kimura 2-parameter model), which showed the anticipated EEHV identity of all EEHV-positive samples found in this study. Labels indicate animal number, type of sample, and date of collection.

6.3.2 Serological status of individuals

Stored serum samples were available for retrospective study. The samples were collected using a consistent methodology, but the frequency of collection was variable for individuals, therefore the number of total samples varied in each individual (Table 6-2). All serum samples were tested for antibody against EEHV1A infection using gB-specific capture ELISA. Results showed variations in serostatus for each elephant over time, throughout the period of the study. The overall serostatus for each elephant was classified according to the criteria set by Angkawanish et al. (2016) and presented in Table 6-2.
Table 6-2. A number of samples tested for antibody against EEHV1A infection using gB-specific capture ELISA. ELISA result and overall serostatus are indicated.

<table>
<thead>
<tr>
<th>Elephant no.</th>
<th>Year of collection</th>
<th>No. of samples</th>
<th>Result (pos:incon:neg)</th>
<th>Overall serostatus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2012 - 2014</td>
<td>8</td>
<td>5 : 3 : 0</td>
<td>OP 5/8 (63%)</td>
</tr>
<tr>
<td>2</td>
<td>2005 - 2015</td>
<td>89</td>
<td>72 : 15 : 2</td>
<td>RP 72/89 (81%)</td>
</tr>
<tr>
<td>3</td>
<td>2012 - 2014</td>
<td>6</td>
<td>0 : 1 : 5</td>
<td>RN 5/6 (83%)</td>
</tr>
<tr>
<td>5</td>
<td>2005 - 2015</td>
<td>89</td>
<td>1 : 31 : 57</td>
<td>ON 57/89 (64%)</td>
</tr>
</tbody>
</table>

Note: Overall serostatus was determined according to the following criteria by Angkawanish et al. (2016): remain positive (RP), when ≥ 80% of all samples were seropositive; occasionally positive (OP), when the number of seropositive samples was < 80%, but the number of seropositive combined with inconclusive samples was ≥ 50% of all samples tested; remain negative (RN), when ≥80% of all samples were seronegative; and occasionally negative (ON), when ≥50% (but < 80%) of all samples were seronegative.

6.3.3 Association between viral detection and serostatus

Serological status varied according to sample timing, therefore only serological results obtained during September 2014 to April 2015 (when molecular testing was also conducted) were considered for this evaluation. Three EEHV viremia episodes were detected in Elephant 1, which was seropositive. Neither EEHV shedding nor viremia was detected in samples collected from Elephant 2, which was consistently seropositive throughout the study period. Two types of EEHV infection were detected in Elephant 3, which was consistently seronegative for EEHV1A infection. Elephant 5 had one episode of EEHV shedding detected, showing borderline levels of antibody titre against EEHV1A...
infection during that period of time. Details of serological and molecular results are presented in Table 6-3. If the inconclusive/borderline level of antibody titre is counted as seropositive, the association between serological and molecular results using the methodology of van den Doel et al. (2015) was found to be 50% in this study.

Table 6-3. Serostatus based on gB-specific ELISA test of each elephant (except Elephant 4) when samples were collected for qPCR testing (September 2014–April 2015). Date when EEHV was positive on qPCR is indicated.

<table>
<thead>
<tr>
<th>Elephant no.</th>
<th>Serostatus</th>
<th>qPCR results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Positive</td>
<td>Pos – EEHV1A (Nov14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pos – EEHV1A (Dec14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pos – EEHV1A (Feb15)</td>
</tr>
<tr>
<td>2</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>Undetectable</td>
<td>Pos – EEHV4 (Sep14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pos – EEHV1A (Feb15)</td>
</tr>
<tr>
<td>5</td>
<td>Inconclusive/Borderline</td>
<td>Pos – EEHV1A (Oct14)</td>
</tr>
</tbody>
</table>

6.3.4 *Faecal corticosterone metabolite*

Fluctuations in faecal corticosterone metabolite (FCM) levels throughout the study period were observed in every elephant. There was also an increasing trend in FCM concentrations, for all individuals, towards the end of this study (Fig. 6-4). However, no major environmental or management changes, or other potentially stressful events, were recorded during the study period. The average FCM concentration from all elephants in this study was $29.79 \pm 9.39$ ng/g ($n = 55$). The average FCM level for each individual is listed in Table 6-4.
**Figure 6-4.** Fluctuations in FCM concentration levels observed in individual elephants. An increasing trend in FCM concentration levels in each elephant towards the end of the study period was observed, however no EEHV DNA was detected at that time.

![Graph showing fluctuations in FCM concentration levels for five elephants over time](image)

**6.3.5 Association between faecal corticosterone metabolite and EEHV detection**

When FCM concentrations from elephants which tested positive for EEHV were compared, three of five EEHV-positive results (60%) occurred when the FCM was lower than the individual’s average FCM levels. The other two positive results (40%) occurred when the FCM was higher than the individual’s FCM average levels (Table 6-4). The association between FCM level and EEHV detection in each elephant is presented in Fig. 6-5. The highest FCM concentration in each elephant did not seem to be associated with EEHV shedding, as no EEHV DNA was detected in either blood or swab samples at that time (Fig. 6-4).
Table 6-4. Serological results of each elephant during September 2014 to April 2015, when blood samples were also collected and tested for EEHV using qPCR. Number of faecal samples, the average FCM concentration in each elephant, and FCM level when EEHV-positive on qPCR are presented.

<table>
<thead>
<tr>
<th>Elephant no.</th>
<th>Serostatus (Sep’14-April’15)</th>
<th>No. faecal samples</th>
<th>FCM ± SD (ng/g)</th>
<th>FCM when EEHV-positive on qPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Positive</td>
<td>9</td>
<td>30.11 ± 10.13</td>
<td>20.57 (Nov14) ↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>34.99 (Feb15) ↑</td>
</tr>
<tr>
<td>2</td>
<td>Positive</td>
<td>13</td>
<td>28.46 ± 7.34</td>
<td>n/a</td>
</tr>
<tr>
<td>3</td>
<td>Negative</td>
<td>11</td>
<td>31.91 ± 10.05</td>
<td>40.7 (Sep14) ↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>23.24 (Feb15) ↓</td>
</tr>
<tr>
<td>4</td>
<td>n/a</td>
<td>14</td>
<td>30.53 ± 12.53</td>
<td>n/a</td>
</tr>
<tr>
<td>5</td>
<td>Borderline</td>
<td>8</td>
<td>27.39 ± 4.06</td>
<td>22.84 (Oct14) ↓</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>55</td>
<td>29.79 ± 9.39</td>
<td></td>
</tr>
</tbody>
</table>

**Note:** Arrow indicates the increase or decrease in FCM levels on the day that EEHV was positive on qPCR, when compared to the average FCM of each individual. No faecal sample was available from Elephant 1 on December 2015, when EEHV was positive on qPCR for that individual.
Figure 6-5. Faecal samples were collected from each elephant between September 2014 and April 2015, and tested for FCM levels. Variations in monthly FCM levels in each individual were observed.
6.3.6 Relationships between serostatus, EEHV detection, and FCM levels

Three episodes of EEHV1 viremia were detected in *Elephant 1*, which was seropositive. However, no association was found between FCM levels and positive EEHV detection in this elephant. Similar results were observed in *Elephant 3*; this elephant had two episodes of EEHV (EEHV1 and EEHV4) detection, and there was no association found between FCM levels and EEHV detection. However, this elephant was seronegative throughout the study period. Further, an episode of EEHV shedding was reported in *Elephant 5*, which gave an inconclusive/borderline titre of antibody level against EEHV1 infection (Table 6-4). The EEHV shedding in this elephant was detected while FCM levels were lower than the average FCM for this individual (27.39±4.06 ng/g).

6.4 Discussion and conclusion

Latent infection with asymptomatic shedding is a unique characteristic of herpesviruses, which provides a potentially undiagnosed reservoir for virus transmission (MacLauchlan & Dubovi, 2011a). To evaluate hypothesised variables associated with EEHV reactivation and shedding, this study considered the history of previous exposure, contact history, serology status, and stressful events in one captive Asian elephant herd in Thailand; chosen because it contained a survivor of clinical EEHV infection. The selection of the herd was based on unique research opportunities, specifically: the presence of previous EEHV clinical disease in one individual; close contact among herd members which could facilitate transmission; and suitably trained elephants and veterinarians to permit sample collection. Five elephants from this herd were selected and monitored for EEHV infection. One of these elephants (*Elephant 1*) had previously been
clinically infected with EEHV (six months prior to this study being conducted). *Elephant 2* (mother of *Elephant 3*) was a surrogate mother for *Elephant 1*. These three elephants (*Elephant 1, 2, and 3*) were housed together in the nursery area until each calf was weaned. *Elephant 4* also had a surrogate-calf relationship with *Elephant 2*, and used the same nursery area after *Elephant 1* was weaned. *Elephant 5* was selected due to having a history of late term abortion on numerous occasions; previous abortions in elephants have sometimes included evidence of herpesvirus in foetal tissue (Fowler, 2008).

During the study period, EEHV was detected in three of five elephants. A higher frequency of viral detection was found in *Elephant 1*, in which three episodes of EEHV viremia were detected during the study. However, the frequency of EEHV detection was highly variable, and no pattern of viral shedding was observed. The level of EEHV in most episodes ranged between $10^3$ and $10^4$ VGCs/ml of blood or swab solution, and these are within the ranges reported in asymptomatic elephants (Stanton et al., 2013). One episode of high viral shedding levels ($>10^5$ VGCs/ml of trunk swab solution) was detected in *Elephant 5*; however, no consistent EEHV clinical signs were observed, and no viremia was detected in the blood collected on the same day. This high level of viral shedding could potentially be a risk for virus transmission to other elephants. However, since EEHV virus has yet to be cultured, studies on viral physiology, pathogenicity and infectious dose (*in vitro*) are limited.

Three episodes of EEHV1 viremia were reported in *Elephant 1*, which had previously been diagnosed. This elephant was diagnosed with EEHV1A infection in mid-February 2014, was treated intensively with intravenous acyclovir (12 mg/kg, BID for seven days) and then continued with the same dose of oral acyclovir for another seven days, antibiotics, fluids, and vitamin supplementation. At that time, *Elephant 1* had clinical
signs consistent with EEHV infection, including discoloration of mucous membranes, petechial haemorrhage on the tip of the tongue, facial and head swelling, lethargy, and anorexia. Blood viral levels exceeded the level of $10^6$ VGCs/ml, and steadily decreased post treatment, with no virus detected in mid-March 2014. The three episodes of EEHV1 detection during this study period in this individual were from blood samples. Trunk swab samples were also collected and tested at these times, however these were found to be negative. This indicated that the viral shedding detected through the trunk swab samples was not consistent with viremia. Due to the very low levels of virus in the sample, it was unable to obtain a clear chromatogram on nucleotide sequencing for further subtype cluster analysis. Therefore, this study could only confirm that the virus found in the blood of Elephant 1 was the same subtype (EEHV1A) as the one which caused the previous clinical infection. Further analysis is needed to determine similarity in subtype cluster level.

EEHV was also detected in the nasal shedding of Elephant 5, which had a history of late term abortion. Although foetal death associated with herpesvirus infection has been reported in other species, for example cattle with Bovine Herpesvirus 1 infection, horses with Equine Herpesvirus 1 infection, and swine with Suid Herpesvirus 1 infection (MacLauchlan & Dubovi, 2011d); further investigation is needed to determine definitively whether EEHV infection can result in abortion in elephants. This would ideally require EEHV investigation from samples from the aborted foetus, maternal placenta, and maternal blood, using both molecular and serological tests.

Despite the contact history, and despite the fact that viral shedding in nasal, oral, or genital secretion provides the source of infection for other animals, including transfer from dam to offspring (MacLauchlan & Dubovi, 2011d), no virus was detected in the youngest
elephant (*Elephant 4*), a two year old elephant which lived with her seropositive mother and fed on maternal milk. This suggests that the elephant may have been naïve to the virus, or still had protective immunity from the mother. Previous studies have reported that maternal breast milk may protect against low doses of Herpes Simplex Virus (HSV) infection in mice (Kohl & Loo, 1984), and maternal immunity against Equine Herpesvirus infection from colostrum is reported to remain up to 180 days postpartum (Carvalho et al., 2000). Despite this evidence from other species, no serological results were available for this elephant, and passive immunity or maternal immunity against EEHV infection has yet to be studied and reported. There remains a need, therefore, for further investigation into maternal and neonatal acquired immunity against EEHV infection.

It is a common phenomenon that hosts are infected with more than one type of herpesvirus (Ehlers, 2008), and mixed infection of EEHV has previously been reported in both healthy and clinically ill elephants (Seilm-Moy et al., 2015; Zong et al., 2015; Fuery et al., 2016). Mixed infection was also found in this study, in *Elephant 3*. Two episodes of viral shedding (EEHV1A and EEHV4) were recorded approximately five months apart; although at these times no clinical signs consistent with EEHV infection were reported. This and other findings suggest that infection with one type of EEHV is unlikely to protect elephants from infection by other types; and also raises the possibility of ineffective cross-immunity between each type. This hypothesis is similar to the situation observed for HSV1 and HSV2, where partial cross-immunity was found between two types, but was not specific enough to prevent infection between types (Wald & Ashley-Morrow, 2002; Kaneko et al., 2008). For these reasons, it is recommended that type-specific molecular and serological tests are needed to definitively diagnose EEHV infection, and to analyse epidemiological relatedness and further improve health management.
Serological testing using EEHV1A-gB-specific capture ELISA was undertaken in four out of the five elephants, to determine serostatus to EEHV infection. Elephant 2, which was the mother of Elephant 3 and surrogate mother of Elephant 1, was consistently seropositive from 2010. Despite the fact that Elephant 1 and Elephant 3 lived closely with Elephant 2 in the same housing facilities, and EEHV circulation within a herd is suspected to occur, both younger elephants showed variable serostatus. Elephant 3 was consistently seronegative (5/6 occasions), and Elephant 1 was occasionally seropositive (5/8 occasions). This variation in serostatus in each elephant, given they lived in a herd with probable circulating EEHV, suggests that immune response to EEHV infection may rely in part on individual immunity and infectious dose of viral shedding (MacLauchlan & Dubovi, 2011b). This hypothesis was supported by the finding of positive antibody titres in Elephant 1 during testing that had been conducted since 2012, which was prior to clinical EEHV infection in this individual in 2014. This finding indicates that the clinical presentation in this elephant was not due to novel exposure, but likely due to the imbalance of host immune-viral control at that particular moment (Grinde, 2013).

As noted above, this study reported a lack of association between the results of serology and molecular tests, using analyses as per van den Doel et al. (2015). Although those authors reported that around 80% of results could be linked by matching these two diagnostic methods, this present study found only 50% relatedness between the two diagnostic tests. This result may be confounded, however, due to seropositive Elephant 2, for which no EEHV DNA was detectable. This was most likely due to intermittent reactivation and shedding of the virus. Importantly, the results of this study demonstrate therefore that negative results from molecular testing may indicate merely a non-shedding period at that specific sampling time – and more frequent sampling efforts may be
required. Another possible reason for the observed results may be a limitation of the qPCR assay in this study. Although a universal EEHV primer was used, this primer has been reported to have greater sensitivity to detect EEHV1 than to detect other EEHV types (Latimer et al., 2011; Zong et al., 2015). Therefore, Elephant 2 may have been infected with types of virus other than EEHV1, which could cross-react and lead to a positive serological result that the qPCR assay used in this study would not detect.

Lack of association between molecular and serological tests was also demonstrated when EEHV DNA was detected in an elephant which was seronegative on ELISA (Elephant 3). This finding was similar to those in the study by van den Doel et al. (2015), where 24% of PCR-positive animals were seronegative. Reasons for this finding may include: i) the elephant was in an early stage of an initial EEHV infection, with specific humoral immunity (particularly IgG) requiring time to develop (Wilson et al., 2006); ii) this elephant was in an early stage of recurrent infection/viral reactivation, while only low levels of specific immunity are needed to control viral replication, and this was too low to be measured (Grinde, 2013); or iii) the lower limit of detection of the ELISA test (cut-off value) was too high.

In humans, eight different types of herpesvirus have been reported, and almost 100% of humans are infected with at least one type of herpesvirus (Grinde, 2013). During latency, the viral genome is retained, and controlled mainly by host immunity (MacLauchlan & Dubovi, 2011b; MacLauchlan & Dubovi, 2011c; Grinde, 2013). Therefore, latency typically results in asymptomatic infection. In humans, a high frequency of herpesvirus reactivation has been reported in immunocompromised patients; such as patients receiving immunosuppressive treatment for cancer therapy (Bustamante & Wade, 1991), or patients with acquired immunodeficiency disease (Sodroski et al., 1987). Similarly, it
may be hypothesised that reactivation of EEHV infection is likely to be triggered by stressful events, such as pregnancy, weaning, husbandry, and management changes; with such stress impeding immune function (Khansari et al., 1990). A previous study of Asian elephants has reported that glucocorticoid levels vary throughout the pregnancy period, with an increasing trend of this hormone during the last month of pregnancy (Kajaysri & Nokkaew, 2014). If stress does induce EEHV reactivation, it would expect to observe more viral shedding and reactivation in the last month of pregnancy. However, one previous study which investigated this hypothesis reported no difference in frequency of EEHV infection throughout the pregnancy period, and also reported a lack of correlation between stress levels and frequency of EEHV detection (Bennett et al., 2015a). This finding fits with those of this study, in which EEHV detection was not consistently correlated with an increasing or decreasing level of the stress hormone.

Glucocorticoid is a stress-induced hormone often used to determine stress levels in both humans and other animals (Möstl & Palme, 2002). To avoid stress during sample collection, non-invasive samples are recommended for analysis (Wasser et al., 2000). As such, this study elected to use non-invasive faecal glucocorticoid sampling; and monitored the concentration levels of FCM to determine stress levels in each elephant during the study. Fluctuations in FCM concentration levels were observed, however when compared with previous studies, FCM concentrations of elephants in this study were within the normal lower ranges, 40–90 ng/g, throughout the study period (Kajaysri & Nokkaew, 2014). The normal lower range of FCM levels was consistent with the lack of stressful events reported throughout the study period. Despite the normal range of FCM, six episodes of EEHV reactivation were detected. This finding suggests that EEHV is spontaneously reactivated and sporadically shed, even when there is a lack of stress.
However, whether stress may increase the frequency of EEHV reactivation remains unknown, and this question requires further investigation.

This longitudinal study monitored EEHV shedding patterns and analysed variables associated with EEHV detection, including type of sample, sampling duration, sampling frequency, and diagnostic testing (SYBR Green I-based real-time PCR and gB-specific ELISA). Sneezing and short distance droplet spread are major modes of herpesvirus transmission in domestic and closely confined animal populations (MacLauchlan & Dubovi, 2011d). Likewise in a captive elephant herd, aerosol spread and close mucosal contact would be expected to be the most likely route of transmission. For these reasons, trunk and conjunctival swabs were chosen to detect viral shedding, and blood sampling was used to determine viral reactivation. Samples were collected every two weeks for seven months (except the blood samples which were collected every month), based on sample collection permission granted by the elephants’ owners.

Sampling frequency and the duration of sampling may affect EEHV detection, as the virus is reactivated and shed intermittently. As per the owner’s request, blood samples were collected monthly while swab samples were collected biweekly; and the frequency of EEHV detection was 7% (6/82). Stanton et al. (2013) reported a 31% (27/87) frequency of EEHV detection when samples were collected from five elephants, one to three times per week for three months, while Hardman et al. (2012), reported a 4% (12/289) frequency of EEHV detection when samples were collected weekly from six elephants for 11 weeks. Low frequency of detection in this study compared to Stanton et al. (2013) suggests collecting samples more frequently could enhance the rate of detection. However, if samples cannot be collected this frequently, as is often the case due to
management-related and logistical issues, sampling duration should be extended, and multiple types and sites of sampling are recommended.

This study found no significant difference in detection of EEHV from blood and trunk swab samples (OR = 4.61; 95%CI: 0.79–26.96; p = 0.067). Previous studies have reported a high percentage of EEHV detection using trunk wash sampling (Stanton et al., 2010; Bennett et al., 2015a). This is likely due to the large volume of samples that can be obtained from this method, which increases the likelihood of viral detection. However, trunk wash collection requires well-trained elephants, which have been trained specifically for trunk wash collection, and the personnel conducting this procedure also need to be well trained, to minimise the risk of zoonotic disease such as tuberculosis (Stephens et al., 2013; Vogelnest et al., 2015). For these reasons, this study elected not to sample using the trunk wash method; and used either trunk swabs or conjunctival swabs instead. These two types of samples were previously reported as the potential sample sources for EEHV detection (Hardman et al., 2009; Sariya et al., 2012).

Choosing the appropriate diagnostic tool is crucial to disease diagnosis. Molecular-based diagnostic testing is a valuable tool for disease monitoring, especially in clinical cases, as the viral levels can be measured alongside clinical disease progression. However, applying this technique to EEHV monitoring in healthy elephants reveals limitations. Conventional PCR lacks analytical sensitivity for the diagnosis of latently infected individuals when only a low number of virus is present, requiring the development of real-time PCR (Stanton et al., 2010; Hardman et al., 2012). Moreover, due to intermittent viral shedding and reactivation, samples need to be collected serially for a defined period of time to increase the chance of viral detection (Stanton et al., 2010; Hardman et al., 2012; Stanton et al., 2013; Bennett et al., 2015a; Long et al., 2015). To monitor effectively
for EEHV, given that multiple samples are needed; and significant monetary funding and veterinary support are also required.

Serological testing is another useful diagnostic tool that can indicate a history of previous exposure. However, as demonstrated in this study, fluctuations in antibody levels can be considerable. As such, multiple samples are required to determine the status of EEHV infection in a single animal. Currently, there is a lack of literature reporting cross-immunity between each type of EEHV. Therefore, a type-specific serological test is required, in order to prevent misinterpretation.

Both serology and molecular tests have their limitations; meaning that multiple tests run in parallel and multiple sampling are required to identify the status of EEHV infection in each individual. According to the guidelines from the EEHV Advisory Group and AZA Elephant SSP, a trunk wash sample should be collected one to two times per week for at least two months, to identify shedding elephants. However, this sampling frequency may not be possible in some circumstances, for example when there are large numbers of elephants within a herd and the budget is limited. This study collected samples every two weeks, as per the elephant owner’s request. Despite this lower frequency of sample collection, the study was still able to detect viremia and viral shedding. Given the large variations amongst results (both molecular and serological), it is recommended that future studies should focus on paired swab and blood samples for qPCR and serology, to identify the virus associated with seropositive individuals (if shedding at the time), which this study was unable to clarify. Although this study was unable to identify an association between EEHV shedding and stress, further studies (where stressful events happen to occur) are required to clarify the relationships between stress and increased frequency of EEHV shedding, and to determine the likelihood of stress-induced EEHV reactivation.
This is the first longitudinal study that has been conducted in an EEHV-positive herd in Thailand. The study has provided new information about patterns of shedding in a captive herd containing an individual survivor of clinical EEHV infection. These findings will be of particular interest to zoo and wildlife veterinarians and elephant conservation groups, and will inform ongoing management of herds containing EEHV survivors. The limitations of this study’s findings are acknowledged with regard to small sample size. The small size of the elephant group limited the statistical analyses available; meaning that results from this study were largely descriptive. It is recommended that further studies are undertaken, based on epidemiological sample sizes determined through power analysis to allow the application of robust statistical tests; to account for issues of intermittent viral shedding and limitations of available tests (O’Brien et al., 2009).

In conclusion, monitoring for EEHV presents several challenges, given that herpesviruses have the property of latency and can shed intermittently. There is currently no one single diagnostic test which can be used to identify an EEHV-free elephant, and no single serological test which can differentiate between past exposure, latency or active infection. Therefore, to maintain healthy elephant populations, multiple tests and serial samples are recommended in each EEHV monitoring plan, and multiple samples from various sites should be collected to increase the likelihood of EEHV detection. Further studies with a robust sample size, including both numbers of elephants sampled and sampling frequency, are required to draw conclusive information about EEHV infection latency and shedding patterns.
CHAPTER 7

GENERAL CONCLUSIONS

AND FUTURE DIRECTIONS
7.1 The future of Asian elephant conservation

In 1986, the Asian elephant was listed as ‘Endangered’ in the IUCN Red List of Threatened Species (IUCN, 2008); indicating that the species faces a high risk of extinction in the near future. The total population of Asian elephants globally is estimated to be approximately 40,000–50,000 (Choudhury et al., 2008). Despite conservation efforts to date, the overall population of Asian elephants continues to decline (Choudhury et al., 2008). This indicates the needs for effective short and long-term conservation strategies to both secure and increase numbers of the remaining populations.

Remaining populations of Asian elephants can be divided into two categories: wild and captive. The conservation threats and challenges facing each group are vastly different. For wild populations, the major threats are habitat loss and modification, and fragmentation of remaining habitat, which separates small populations and poses a serious threat to genetic fitness and health (Choudhury et al., 2008). Moreover, the decrease in habitat has reduced foraging areas, forcing Asian elephants into surrounding, human-occupied areas, which has in turn increased human-elephant conflicts. Other threats include hunting, ivory trading, and disease. The expansion of urban areas may increase the likelihood of disease transmission between wild elephants and domestic animals (i.e. encephalomyocarditis virus, foot and mouth disease), both of which could lead to the decline of the wild elephant population (Fowler, 2008). These issues all have the potential to lead to the extirpation of wild Asian elephant populations; and although recent conservation efforts have attempted to reduce habitat loss, poaching, and the ivory trade, these threats are ongoing. Further, human-elephant conflicts, on the other hand, are reportedly increasing (Hedges, 2006). This is despite management efforts including; the establishment of buffer zones or physical barriers, such as fences, between elephant
habitat and human agricultural areas; human patrolling; and elephant translocations. Long term management solutions are urgently needed, with appropriate levels of financial and other commitments to ensure conservation management success.

Compared to wild population, captive Asian elephant populations face somewhat different threats. Asian elephants have been kept in captivity in sizeable numbers historically across their range, and have played an important role in local culture, tradition, and human life. Therefore, a large proportion of the surviving Asian elephant population in each range country is now in some form of captivity. In Thailand for example, almost half the remaining Asian elephant population is in captivity (Angkawanish et al., 2009). Currently, the number of Asian elephants in captivity across all range countries is reportedly self-sustaining or slightly increasing (Hedges, 2006). However, despite their present stable population size, captive populations remain threatened by poor reproductive performance, a biased sex ratio, and frequent health problems (especially foot problems) (Hedges, 2006; Mikota & Fowler, 2006; Veasey, 2006). Captive Asian elephants also face serious welfare issues, indicating a need for appropriate, standardised management and welfare guidelines supported by legislation and community education; along with adequate monitoring and law enforcement to safeguard the welfare and long-term viability of captive populations (Veasey, 2006; Carlstead et al., 2013). Ensuring the viability of captive elephant populations will provide a range of conservation benefits, including the provision of animals for conservation-focused research for extrapolation to wild populations; and a security population as wild populations continue to decline. Research into the welfare, health and care of captive Asian elephant populations is therefore critical for conservation of the species.
In Thailand, despite some effort to address Asian elephant conservation, including new laws protecting areas of natural habitat as well as laws prohibiting illegal hunting and the ivory trade, wild Asian elephants are still challenged by habitat fragmentation (Thitaram et al., 2015). In light of this challenge, large-scale elephant translocations or the development of wildlife corridors are likely to be required as conservation management strategies in the near future. In contrast, Thailand’s captive Asian elephant population is self-sustaining, and increasingly driven by the rise in elephant-based tourism. Urgent management requirements for captive elephant populations in Thailand include: (1) improved welfare associated with handling and husbandry of elephants in captive facilities, including stronger monitoring and law enforcement of welfare standards; (2) effective education about welfare and conservation needs, guidelines and legislation, both within captive elephant facilities and at the broader community level; (3) a reliable, adequately-resourced registration system for individual elephants and breeding management, in order to reduce illegal hunting and illegal capture of wild elephants for captivity; and (4) appropriate herd health management and, health management of individual captive elephants.

7.2 Current situation with regards to clinical EEHV infection and the limitation of diagnostic tests

EEHV infection can cause fatal haemorrhagic disease in young elephants, particularly Asian elephants. The reports and potential impacts of EEHV HD have driven increased awareness and interest in elephant conservation and research, aimed at exploring pathogenesis and the development of therapeutic interventions. Despite attempts to date,
the virus has yet to be cultured and grown in vitro. Therefore, the majority of information regarding EEHV has been obtained from clinical case histories, molecular evidence, and extrapolation from other closely related herpesviruses.

The failure to culture the virus has prevented development of a vaccine against EEHV infection. In the absence of a vaccine, disease monitoring has been instituted to reduce the number of fatalities (Long et al., 2015). Both conventional PCR and quantitative real-time PCR are commonly used for disease monitoring in asymptomatic elephants, with application of these techniques enabling the disease to be detected in its early stages, thereby improving the likelihood of treatment success (Wiedner et al., 2012). Serological tests using ELISA assays also provide information regarding immune status of individuals and herds, and this is important to improve disease management plans (van den Doel et al., 2015).

While disease monitoring systems may help to reduce the occurrence of EEHV, well-prepared and resourced laboratories, appropriate treatment protocols, and sufficient public awareness are also required. To date, only three countries in Asia (Thailand, India, and Indonesia) have EEHV diagnostic facilities. Greater numbers of diagnostic facilities are required in Asian elephant range countries to ensure effective monitoring for this disease. Adequate training of elephants to facilitate sampling is also very important, as is the provision of training to mahouts and elephant keepers to observe the early signs of EEHV infection. Given the size of Asian elephants and the large quantities of medicine required for treatment, advanced preparation of drugs and advance contact with drug distributors are also required to enable timely treatment. Together, these aspects of disease preparedness are vital to minimise clinical EEHV infection.
7.3 Key findings from this study

- At least 18 cases of clinical EEHV infection have been confirmed in Thailand in the past decade, as presented in this study. This is the largest number of confirmed cases of EEHV HD reported in an Asian elephant range country.

- 16 of the 18 aforementioned elephants in this study, which presented with clinical signs of EEHV, succumbed to the disease. The disease occurred most frequently in young elephants (particularly in juveniles, ages 1–5 years old). This indicates the urgent need for disease monitoring and management in this age class.

- Based on multiple gene analysis, a large variety of subtype clusters were found among positive cases. None of the viruses recovered from each positive case were identical over multiple gene loci. This suggests that the disease is likely to be sporadic, and the virus is likely to be an endogenous pathogen of Asian elephants. These findings are analogous to the findings of Zachariah et al. (2013). The present study also showed the importance of using multiple gene analysis for epidemiological research.

- No history of direct contact between Asian and African elephants was recorded in positive EEHV HD cases in Thailand. Therefore this study’s findings do not support the hypothesis that the disease was transmitted via cross-species transmission (Richman et al., 1999). This concurs with the findings of Zachariah et al. (2013) whereby EEHV1A and 1B, at least, are proposed to be natural endogenous pathogens which have coevolved with Asian elephants over long periods of time. Moreover, based on genetic analysis Probosciviruses evolved separately from all other mammalian herpesviruses approximately 100 million years ago, when the ancestors
of modern elephants split from all other placental mammals about 35 million years ago (Zong et al., 2014). This suggests that EEHV is an old virus that is likely to have coevolved with elephants.

- This study selected universal PANPOL primers and applied the SYBR green I-based real-time PCR assay to identify subclinical infection, to diagnose early stages of infection, and to monitor disease progression. Results indicated that this technique provides sufficient data, with appropriate detection limits. This technique also can differentiate at least three different types of EEHV (EEHV1A, 1B, and 4).

- Application of newly developed real-time PCR technique to undertake EEHV screening in healthy elephant populations in Thailand indicates that the apparent prevalence of asymptomatic EEHV1 infection in captive Asian elephants in Thailand was 5.5% at the time of this research.

- This is the first study to report EEHV infection in asymptomatic Asian elephants in Thailand. This research did not find that sex, geographical location, or sample site were related to EEHV detection. However, juvenile elephants were more frequently detected with the virus, compared to other age classes combined (OR = 4.46; 95% CI: 1.60–12.45; p = 0.05).

- Preliminary testing for the presence of EEHV in semen samples from nine elephant bulls produced negative results.

- EEHV virus is shed and reactivated intermittently; given which, the results from the cross-sectional study undertaken as part of this research provided information on the overall prevalence of EEHV infection during the period of study, but did not provide
information about the infection status of individual elephants. To determine the status of infection in individuals, serial sampling is required.

- This research included the first longitudinal study in an EEHV-positive herd in Thailand. The study provided new information about patterns of shedding in a captive herd containing an individual survivor of clinical EEHV infection. This study demonstrated that the frequency of EEHV detection varies both within and between individuals. The study also found a significant difference between detecting EEHV in an elephant which had survived EEHV infection six months prior to the commencement of this study, compared to other elephants in the study (OR = 4.85; 95% CI: 0.88–26.74; p = 0.05).

- In the longitudinal study, despite the elephants having a normal range of faecal corticosterone metabolite, six episodes of EEHV reactivation were detected. This finding suggests that EEHV is spontaneously reactivated and sporadically shed, even when there is a lack of stress. However, it is unknown if stress can contribute to a higher shedding frequency, therefore further investigation is required.

- A lack of association between molecular (qPCR) and serological (ELISA) tests was found; and the results for each test also varied depending on the sampling time. Therefore, to identify EEHV status in asymptomatic elephants, serial sampling is required, and the use of multiple diagnostic tools is recommended. It should be noted that negative results (from either PCR or ELISA) from one particular time (i.e. from a single sample) are not sufficient to claim that an animal is free from EEHV infection.
- This study found no significant difference in the detection of EEHV from blood or swab samples (n = 82, OR = 4.61; 95% CI: 0.79–6.96; p = 0.067), and as such, this study cannot recommend one sample type over another. However, it is recommended that multiple types of samples should be collected, to increase the chance of viral detection; although this is dependent on the clinical skills of the elephant keeper and elephant compliance.

- Frequent sampling provides a better chance of detecting infection. However, it should be noted that if samples cannot be collected frequently, a longer sampling period is recommended.

- This study is the first cross-sectional and longitudinal EEHV study to be conducted in an Asian elephant range country, and logistical and management-related issues affecting sample size and frequency were apparent. This study found asymptomatic EEHV infection in captive elephants in Thailand, illustrating that latent infection was possible, and healthy elephants can carry this virus. The findings from this present study together with evidence of the long coevolution of virus and host suggest that the EEHV virus is likely to exist in most, if not all, of the Asian elephant’s range countries. A lack of information from some countries may be due to limitations associated with diagnostic facilities and a lack of public awareness. Further collaboration to manage the threat of EEHV, both internationally and locally, is urgently needed.

- The research findings from the longitudinal study were limited by a small sample size; and the small size of the elephant group limited the statistical analyses. It is recommended that further studies are based on epidemiological sample sizes
determined through power analysis to allow the application of robust statistical tests, to account for issues of intermittent viral shedding and limitations of available tests.

- Choosing the diagnostic test is one of the important steps in disease monitoring, as each test has its own limitations. Molecular diagnostic tests (i.e. PCR and qPCR) can only be used to identify animals with active infection or when viral reactivation. This means that PCR testing with only one-shot sampling cannot used as a screening tool for potentially infected latent carrier animals. Whereas serological tests (i.e. ELISA) could indicate previous exposure or potential carrier status, however, serology is more useful when undertaken in conjunction with PCR for diagnosis and evaluation of the immune response to active infection as well as monitoring the levels of exposure to EEHV, period of detectable antibodies, and the general epidemiology of the disease itself.

7.4 Further research directions and management recommendations

Factors associated with EEHV shedding/reactivation

Although EEHV is shed spontaneously from asymptomatic elephants, factors which increase shedding frequency remain unknown. Identifying the factors associated with EEHV shedding would be of great benefit to disease management and prevention. This study found that fluctuations in glucocorticoid levels (within the normal range) did not affect the frequency of EEHV shedding (see Chapter 6, Section 6.3.5). However, the question as to whether certain stressful events (which increase stress levels to above normal levels) may increase the frequency of EEHV shedding remains unknown, and
requires further investigation. It is recommended that EEHV reactivation and stress levels should be monitored in captive Asian elephants before, during, and after they are subjected to any potentially stress events; for example translocations, weaning, changes in herd status, and major changes to management or environment. In addition to monitoring the frequency of shedding, viral numbers should also be monitored, to better understand viral dynamics during stress events.

Blood profile changes may also be associated with viral reactivation, and these values should be monitored in parallel to any events suspected to promote viral reactivation. Asymptomatic shedding of Herpes Simplex Virus 1 (HSV1) was reported to increase in frequency, in patients with severe cytopaenia (Sepúlveda et al., 2008); similar to the reactivation of Cytomegalovirus (CMV) observed in immunosuppressed patients (Emery, 2001). This study was unable to monitor elephants’ blood profiles during EEHV surveillance due to budget constraints. However, it is recommended that whenever possible, blood profiles (particularly white blood cell count) should be monitored alongside EEHV testing.

**Maternal and neonatal immunity**

Herpesviruses generally only cause mild symptoms; however, when herpesvirus infections occur during the neonatal period, viral replication is poorly controlled by host immunity, and severe effects are commonly observed (Gantt & Muller, 2013). Changes to the immune system during the transition from foetal to post-natal life leave animals particularly vulnerable to infection (Gantt & Muller, 2013). This first acute transition is then followed by a gradual, age-dependent maturation of the immune system (Levy,
Therefore, the ability of a neonatal elephant to mount an effective immune response relies largely on maternal immunity and innate immunity.

According to the findings from this study (see Chapter 3) and from previous research, EEHV-associated fatalities occur most commonly in young elephants (Hayward, 2012; Richman & Hayward, 2012). Adult elephants are thought to serve as a reservoir, and present with only mild symptoms or subclinical infection. The reasons why the disease is more severe in young elephants, and why some young elephants become infected without developing clinical signs, are still unknown. Further research into immune responses to EEHV infection for all age classes are required.

Neonatal fatalities associated with herpesvirus infection are also reported in humans (Gantt & Muller, 2013). Approximately 85% of neonatal HSV2 infection is acquired during the passage through the birth canal, while 10% of HSV2 infections are acquired post-partum (via care-givers), and 5% in-utero (Gantt & Muller, 2013). The severity of HSV infection in neonates is increased significantly for those whose mothers had a primary HSV infection during late gestation (Brown et al., 1987). This is due to the limitations of transplacental immunity transferred to the neonate (Prober et al., 1987; Arvin, 1991). Clinical signs for HSV2 infection in neonates are greater in severity when the mother is immunologically naïve to HSV1. Lack of maternal HSV1 exposure means the infant receives no transplacental immunity from HSV-specific antibodies that could partially protect against other types of HSV (Brown et al., 1987).

Congenital CMV infection in humans provides further evidence of the importance of maternal immunity in protecting neonates against herpesvirus infection. CMV belongs to the Betaherpesvirus group (similar to EEHV), and infection with this virus leads to significant neurological disabilities in human infants (Schleiss & McVoy, 2010). This
virus is transferred from mother to foetus both vertically (during pregnancy or delivery) and horizontally (via breast milk) (Mestas, 2016). Up to two percent of pregnancies show \textit{in-utero} transmission of CMV from mother to foetus (Schleiss & McVoy, 2010). Approximately 10% of babies born with CMV infection will be symptomatic at birth, with the remainder developing symptoms during the first five years of life (Mestas, 2016).

CMV infection is similar to HSV infection in that maternal immunity decreases the frequency of transplacental transmission of the virus, and the presence of maternal immunity before conception can also provide substantial protection against the effects of CMV infection in newborns (Fowler et al., 1992; Fowler et al., 2003). However, we noted that this elephant herpesvirus has unique genetic characterisations, and has been proposed to classify as a new subfamily of herpesviruses (Richman et al., 2014). Therefore caution is required when comparing this virus to other mammalian herpesviruses.

As described, passive immunity plays a pivotal role in neonatal immunity, in protecting an infant from herpesvirus infection or severe pathological effects; and therefore, it is vital to understand the role of passive immunity. Passive immunity, particularly via the maternal antibodies (IgG), is transferred to the infant during pregnancy (via the placenta) and after birth (via colostrum). Both forms provide active antibodies against particular infections. The amount of antibody transferred by these two mechanisms differs by species, and is dependent on the type of placenta (Niewiesk, 2014). In elephants, the placenta is endotheliochorial, which creates a barrier that prevents \textit{in-utero} transfer of immunoglobulins to the foetus (Schmitt, 2006). In elephants, therefore, passive immunity is acquired mainly from colostrum; and needs to be obtained ideally within the first six hours and no later than 24 hours post-partum, to allow immunoglobulin absorption (Emanuelson, 2006). The persistence of maternal immunity varies by species. In humans,
maternal immunity lasts for between six to 12 months; in many domestic livestock it lasts for three to six-months; whilst in chickens it persists only for four to seven days (Niewiesk, 2014). As passive immunity in elephants relies so heavily on maternal colostrum, research is urgently needed to investigate the components of colostrum, especially the immunoglobulin components, relevant to the defence against EEHV. This information would be of major benefit in the development of effective prevention plans and management guidelines to control this disease.

**Serological testing**

To understand the epidemiology and the pathophysiology of EEHV in elephants, it is critical to understand the host’s immune response. This will have an impact on case management, surveillance and an improved knowledge of epidemiology. Surveillance requires active assessment of virus presence combined with accurate serological testing. However, it is challenging to develop a robust serological test in the absence of viral isolation. Serological testing is a useful tool to detect asymptomatic infection and active cases; however, it is likely that this test cannot be applied within the fatality cases, as these animals likely did not mount antibody against EEHV infection. The first serological test for EEHV infection has recently been developed, based on molecular genetic information and the properties of other herpesviruses (van den Doel et al., 2015). Preliminary results of the test’s performance with EEHV1 infection indicated that the serological test has a good ability to detect the virus (van den Doel et al., 2015). However, the lower limit of detection of this test is still relatively high, due to issues related to contamination of the coated antigen. Moreover, given that seven types of EEHV have been reported to date, and mixed infection with different types of the virus has been observed in both clinical and subclinical elephants, the ability of a test to differentiate
between types is important (Zong et al., 2015; Fuery et al., 2016; Seilern-Moy et al., 2016). This study also found mixed infection with no different in serological response (see Chapter 6, section 6.3.2 and 6.3.3). Therefore, there remains a need for a type-specific serological test, to assist with investigating immune responses to each EEHV type, and to investigate the disease’s epidemiology.

Serological tests which differentiate between herpesvirus types can be particularly beneficial for herpesviruses, which exhibit different pathogenicities by viral types. In humans, a type-specific serological test for HSV infection is widely used for asymptomatic diagnosis. The identification of the type of HSV provides great benefits for disease management, because the specific type of HSV infection affects disease prognosis and subsequent patient lifestyle counselling (Wald & Ashley-Morrow, 2002; Strick & Wald, 2004). Like HSV, different types of EEHV exhibit differences in pathogenicity. EEHV1 is the most commonly diagnosed EEHV viral type found in Asian elephants; while EEHV 3 and 4 are rarely diagnosed but tend to cause severe clinical disease (Garner et al., 2009; Sripiboon et al., 2013). EEHV5, in contrast, is frequently found in asymptomatic elephants, but rarely contributes to clinical disease (Atkins et al., 2013; Stanton et al., 2014; Wilkie et al., 2014). Type-specific serological results are recommended to assist disease management, and also to facilitate epidemiological research by identifying viral exposure status within a herd. Moreover, serological tests are an essential tool for vaccine development, to monitor the level of antibody produced post vaccination. There is particular a need for further investigation into the antigenic sites of EEHV, which could stimulate and bind with neutralised antibodies. Such research should include the identification of both type-specific and common antigenic sites.
**Rapid test**

Given the rapid progression of EEHV HD, a fast and reliable diagnostic test is crucial. The recent development of real-time PCR for EEHV diagnosis provides relevant information within a short time frame. However, this technique requires sophisticated laboratory equipment and technical expertise (see Chapter 4). Most captive elephant facilities in Asian elephant range countries occur in remote or regional areas, without ready access to testing facilities and technical laboratory support. Sample storage and transportation may also be challenging. A rapid field-appropriate test, such as a strip test which could be utilised in the field to provide immediate results, would be of great benefit for EEHV treatment and elephant conservation, particularly given the necessity for fast intervention following diagnosis of this disease. However, the lack of ability to culture the virus or harvest virus for testing challenged the development of a rapid test kit. Glycoprotein B is considered to be a potential antigenic site for this virus (Fickel et al., 2003; van den Doel et al., 2015). This protein may therefore be a good candidate for the development of a test kit in the future. However, further research into this and other potential antigenic sites is required.

**Viral culture and vaccine development**

Information about viral diseases, including diagnosis and vaccine development, relies heavily on viral isolation. However, not every virus is able to be cultured. Despite attempts to date, EEHV is yet to be isolated, even with the use of elephant-based cell lines (Latimer et al., 2011; Bennett et al., 2015b; Long et al., 2015). Currently, only limited information is available regarding EEHV isolation efforts. Published reports have tended to describe the lack of success of cultures, without presenting details of laboratory
techniques (Richman et al., 1999; Latimer et al., 2011; Richman & Hayward, 2012). With ongoing advancements in viral isolation, it is possible that EEHV may be cultured in the future. In the meantime, published reports outlining techniques used during culture attempts will be particularly useful to guide subsequent research, and enable researchers to refine these techniques. Based on vaccine development with other herpesviruses, there is a limitation of acute clinical disease caused by herpesviruses, once infection is established, which seems to reside in mechanisms other than antibody response, therefore disease may reoccur in spite of high levels of neutralizing antibody.

A key step in the control of any viral disease is to develop a vaccine. However, the development of an EEHV vaccine remains elusive in the absence of successful attempts to culture the virus. For viruses unable to be cultured, other options include the development of subunit vaccines and recombinant vaccines. However these options require time and multiple trials prior to the production of a commercial vaccine. Despite the challenges associated with development of an EEHV vaccine, the threat which this disease poses underscores the value and urgency of all Asian elephant range countries increasing their EEHV research efforts. It is recommended that research should be focussed on vaccine development and other key aspects of EEHV disease management, for example longitudinal monitoring of infected individuals in herds and monitoring of high disease-risk groups such as young elephants.

**Elephant management recommendations regarding EEHV infection**

Evidence from previous studies and this study suggest that Asian elephants naturally carry at least two subtypes of EEHV: EEHV1A and EEHV1B (Zachariah et al., 2013; Long et al., 2015; Zong et al., 2015) (see Chapter 3 and 5). However, factors contributing to
disease occurrence remain unclear and no vaccination is available, therefore a comprehensive disease monitoring protocol is essential in all Asian elephant herds.

EEHV HD generally occurs in young elephants, particularly juveniles; therefore intensive monitoring should be conducted in this age class. The best way to monitor the disease currently is to use molecular diagnostic testing, however as this technique requires laboratory expertise and financial support, the application of this recommendation into every elephant facility in range countries may be difficult. As a consequence, this study proposes the following management recommendations:

- To combat viral infection, host-immunity is crucial. Although at present there is a lack of evidence to correlate host-immunity with EEHV infection, following the precautionary principle, this study recommends strongly that every infant elephant should receive colostrum and should feed on maternal breast milk, in order to stimulate neonatal immunity.

- Although the factors leading to EEHV HD remain unknown, this study recommends that all young elephants are monitored daily for early signs (such as abnormal body temperature, behavioural change, dullness, anorexia) and EEHV-related clinical signs (such as petechial haemorrhage at the tip of the tongue, oral ulcer); and that intensive monitoring through molecular testing are performed and treatment are planned. Furthermore, during and after potential stress events, for example weaning, transportation, diet change, herd status changes, and any major environmental and management changes; blood and swab samples should be collected weekly for two to three months for EEHV molecular testing.
EEHV-related clinical signs include dullness, anorxia, facial and neck swelling, tongue cyanosis, and petechial haemorrhages at the tip of the tongue. In order to observe these signs, elephants should be trained to facilitate this observation protocol at an early age, and trained to facilitate blood collection and other medical procedures. In addition, a poster displaying photographs of typical EEHV clinical signs is recommended to be placed in every elephant housing facility, to educate and inform elephant handlers. Emergency contact details and an initial treatment protocol should also be included on the poster, to facilitate timely treatment.

It is recommended that standard blood profiles of each individual elephant should be established in each elephant facility, given the variation in blood values amongst individuals and age classes. It is much better to compare blood results to an individual’s own standardised values, than to the age specific normal reference ranges.

Each elephant facility should have the contact information of the nearest laboratory and veterinary services, in order to confirm infection and commence treatment as required, as soon as clinical signs appear.

Availability of information and public awareness about EEHV are crucial aspects of risk management for this disease. The establishment of a focal centre to exchange information (molecular, clinical, diagnostic and epidemiological) within and between countries is also needed, and would greatly benefit elephant conservation through knowledge transfer both locally and internationally (available on www.eehvinfor.org). Drug stockpiling, capacity building and technology transfer to rural and remote areas are also needed, to manage the
disease and prevent it from having a major impact on the remaining Asian elephant populations in their home range country.

Understanding the disease status of Asian elephant populations is an essential part of Asian elephant conservation. Disease monitoring and surveillance will play a pivotal role for both wild and captive individuals to enable successful treatment of EEHV HD clinical cases, in lieu of the development of an effective and readily available vaccination. Elephant Endotheliotropic Herpesvirus HD is a sporadic but highly fatal disease in young elephants and has the potential to hasten Asian elephant population declines. It is hoped that information from this study will inform disease management plans to help prevent or lessen the impact of EEHV disease in the future. Elephants are an integral part of Thailand and Southeast Asian society, and we hope that this approach can certainly be extrapolated for use in other SE Asian countries. Elephants are cultural icons, promote tourism, and play an important role in ecosystem health. Research-driven management plans to sustain elephant populations and manage EEHV will have a positive impact not only on Asian elephant conservation, but also on the human communities whose countries they share.
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MacLauchlan, N. J. & Dubovi, E. J. (2011b). Pathogenesis of viral infections and


Tateishi, K., Toh, Y., Minagawa, H. & Tashiro, H. (1994). Detection of herpes simplex virus (HSV) in the saliva from 1,000 oral surgery outpatients by the polymerase chain reaction (PCR) and virus isolation. *Journal of Oral Pathology and Medicine*, 23, 80-84.


APPENDIX 1: The classification of order *Herpesvirales*

<table>
<thead>
<tr>
<th>Taxon.</th>
<th>Name (Acronym)</th>
<th>Common name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Order</td>
<td><em>Herpesvirales</em></td>
<td></td>
</tr>
<tr>
<td>Family</td>
<td><em>Herpesviridae</em></td>
<td></td>
</tr>
<tr>
<td>Subfamily</td>
<td><em>Alphaherpesvirinae</em></td>
<td></td>
</tr>
<tr>
<td>Genus</td>
<td>Simplexvirus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Human herpesvirus 1 (HHV1)</td>
<td>Herpes Simplex virus type 1</td>
</tr>
<tr>
<td></td>
<td>- Human herpesvirus 2 (HHV2)</td>
<td>Herpes Simplex virus type 2</td>
</tr>
<tr>
<td></td>
<td>- Macacine herpesvirus 1 (McHV1)</td>
<td>Herpes B virus</td>
</tr>
<tr>
<td>Genus</td>
<td>Varicellovirus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Human herpesvirus 3 (HHV3)</td>
<td>Varicella-zoster virus</td>
</tr>
<tr>
<td></td>
<td>- Bovine herpesvirus 1 (BoHV1)</td>
<td>Infectious bovine rhinotracheitis virus</td>
</tr>
<tr>
<td></td>
<td>- Canid herpesvirus 1 (CaHV1)</td>
<td>Canine herpesvirus</td>
</tr>
<tr>
<td></td>
<td>- Felid herpesvirus 1 (FeHV1)</td>
<td>Feline rhinotracheitis virus</td>
</tr>
<tr>
<td></td>
<td>- Equid herpesvirus 1 (EHV1)</td>
<td>Equine abortion virus</td>
</tr>
<tr>
<td></td>
<td>- Suid herpesvirus 1 (SuHV1)</td>
<td>Pseudorabies virus</td>
</tr>
<tr>
<td>Genus</td>
<td>Iltovirus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Gallid herpesvirus 1 (GaHV1)</td>
<td>Infectious laryngotracheitis virus</td>
</tr>
<tr>
<td></td>
<td>- Psittacid herpesvirus 1 (PsHV1)</td>
<td>Pacheco’s disease</td>
</tr>
<tr>
<td>Subfamily</td>
<td><em>Betaherpesvirinae</em></td>
<td></td>
</tr>
<tr>
<td>Genus</td>
<td>Cytomegalovirus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Human herpesvirus 5 (HHV5)</td>
<td>Human cytomegalovirus</td>
</tr>
<tr>
<td>Genus</td>
<td>Muromegalovirus</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>-----------------</td>
<td></td>
</tr>
<tr>
<td>Genus</td>
<td>Roseolovirus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Human herpesvirus 6 (HHV6) Human herpesvirus 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Human herpesvirus 7 (HHV7) Human herpesvirus 7</td>
<td></td>
</tr>
<tr>
<td>Genus</td>
<td>Proboscivirus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Elephantid Herpesvirus (EEHV) Elephant Endotheliotropic Herpesvirus</td>
<td></td>
</tr>
<tr>
<td>Subfamily</td>
<td>Gammaherpesvirinae</td>
<td></td>
</tr>
<tr>
<td>Genus</td>
<td>Lymphocryptovirus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Human herpesvirus 4 Epstein-Barr virus</td>
<td></td>
</tr>
<tr>
<td>Genus</td>
<td>Rhadinovirus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Human herpesvirus 8 Kaposi’s sarcoma-associated herpesvirus</td>
<td></td>
</tr>
<tr>
<td>Genus</td>
<td>Macavirus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Alcelaphine herpesvirus 1 Malignant catarrhal fever virus</td>
<td></td>
</tr>
<tr>
<td>Genus</td>
<td>Percavirus</td>
<td></td>
</tr>
<tr>
<td>Family</td>
<td>Alloherpesviridae</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Cyprinid herpesvirus Koi herpesvirus</td>
<td></td>
</tr>
<tr>
<td>Family</td>
<td>Malacoherpesviridae</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Ostreid herpesvirus 1 Oyster herpesvirus</td>
<td></td>
</tr>
</tbody>
</table>
**APPENDIX 2: EEHV report form**

**EEHV report form**

<table>
<thead>
<tr>
<th>SECTION 1: Reporter information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name:</td>
</tr>
<tr>
<td>Position:</td>
</tr>
<tr>
<td>Address:</td>
</tr>
<tr>
<td>Email:</td>
</tr>
<tr>
<td>Tel:</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SECTION 2: Elephant general information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elephant’s name:</td>
</tr>
<tr>
<td>Microchip no.</td>
</tr>
<tr>
<td>Date of birth:</td>
</tr>
<tr>
<td>Age:</td>
</tr>
<tr>
<td>Sex:</td>
</tr>
<tr>
<td>Birth place:</td>
</tr>
<tr>
<td>Current location:</td>
</tr>
<tr>
<td>Sire’s name:</td>
</tr>
<tr>
<td>Dam’s name:</td>
</tr>
<tr>
<td>Elephant was born in [ ] captivity [ ] wild [ ] unknown</td>
</tr>
<tr>
<td>Is this elephant still stay with mother [ ] Yes [ ] No</td>
</tr>
<tr>
<td>Do any elephants be housed in the same stable? [ ] Yes [ ] No</td>
</tr>
<tr>
<td>How many (please specify)</td>
</tr>
<tr>
<td>Has this elephant ever interacted with a known EHV-positive elephant before? [ ] Yes [ ] No</td>
</tr>
<tr>
<td>If yes, please describe the relationship between this elephant and known EHV-positive elephant</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Have you ever seen these following signs with your elephant before?</th>
</tr>
</thead>
<tbody>
<tr>
<td>[ ] Oral ulceration (when, please specify)</td>
</tr>
<tr>
<td>[ ] Genital ulceration (when, please specify)</td>
</tr>
<tr>
<td>[ ] Skin nodule (when, please specify)</td>
</tr>
</tbody>
</table>

| Have this elephant gone to any major sickness before? (please describe) |

<table>
<thead>
<tr>
<th>SECTION 3: EEHV related information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date that clinical signs were observed</td>
</tr>
<tr>
<td>What are the clinical signs presented?</td>
</tr>
<tr>
<td>[ ] Dull/depression/lethargy</td>
</tr>
<tr>
<td>[ ] Decrease appetite</td>
</tr>
<tr>
<td>[ ] Head/face swell</td>
</tr>
<tr>
<td>[ ] Petechial haemorrhage at tip of the tongue/tongue cyanosis</td>
</tr>
<tr>
<td>[ ] Lameness</td>
</tr>
<tr>
<td>[ ] Diarrhea</td>
</tr>
<tr>
<td>[ ] Others (please specify)</td>
</tr>
<tr>
<td>Has treatment been conducted</td>
</tr>
<tr>
<td>------------------------------</td>
</tr>
</tbody>
</table>

If no, please describe why treatment cannot be conducted this time

What treatment protocol is used? (drug name, route, frequency, duration)

When treatment is conducted
- [ ] Immediately after clinical signs were observed
- [ ] After clinical signs were observed (please specify how many days after clinical signs were showed)
- [ ] Other

What is the outcome from the treatment
- [ ] survive
- [ ] die

### SECTION 4: Diagnostic

<table>
<thead>
<tr>
<th>Have this elephant been diagnosis</th>
<th>[ ] Yes</th>
<th>[ ] No</th>
</tr>
</thead>
</table>

What kind of samples be sent to diagnosis

Where do you send sample to

Result

Has gross examination been conducted
- [ ] Yes
- [ ] No

If yes, please describe gross results

Has histopathology been conducted
- [ ] Yes
- [ ] No

If yes, is inclusion body visible
- [ ] Yes
- [ ] No

Please describe histopathological finding

### SECTION 5: other comments
APPENDIX 3: Normal blood parameters in Asian elephants

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Ref (^1) (mean ± SD)</th>
<th>Ref (^2) (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Young (≤ 9 years)</td>
<td>Adult (9-30 years)</td>
</tr>
<tr>
<td>WBC</td>
<td>x 10(^3)/µl</td>
<td>19.15±6.16</td>
<td>15.15±4.56</td>
</tr>
<tr>
<td>RBC</td>
<td>x 10(^6)/µ</td>
<td>3.22±0.73</td>
<td>3.00±0.61</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>g/dl</td>
<td>12.5±2.4</td>
<td>12.9±2.0</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>%</td>
<td>36.0±6.2</td>
<td>36.7±5.1</td>
</tr>
<tr>
<td>MCV</td>
<td>fl</td>
<td>113.7±17.8</td>
<td>122.9±13.7</td>
</tr>
<tr>
<td>MCH</td>
<td>pg/cell</td>
<td>40.7±5.8</td>
<td>43.0±5.6</td>
</tr>
<tr>
<td>MCHC</td>
<td>g/dl</td>
<td>34.9±3.3</td>
<td>35.1±2.5</td>
</tr>
<tr>
<td>Platelet count</td>
<td>x 10(^3)/µl</td>
<td>463±205</td>
<td>383±281</td>
</tr>
<tr>
<td>Reticulocytes</td>
<td>%</td>
<td>1.1±1.9</td>
<td>0</td>
</tr>
<tr>
<td>Seg. Neutrophils</td>
<td>x 10(^3)/µl</td>
<td>5.87±3.96</td>
<td>4.96±2.77</td>
</tr>
<tr>
<td>Band Neutrophils</td>
<td>x 10(^3)/µl</td>
<td>2.61±2.58</td>
<td>0.97±1.13</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>x 10(^3)/µl</td>
<td>7.25±4.13</td>
<td>5.92±3.17</td>
</tr>
<tr>
<td>Monocytes</td>
<td>x 10(^3)/µl</td>
<td>3.83±2.99</td>
<td>2.99±2.73</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>x 10(^3)/µl</td>
<td>0.47±0.61</td>
<td>1.23±1.54</td>
</tr>
<tr>
<td>Basophils</td>
<td>x 10(^3)/µl</td>
<td>0.22±0.13</td>
<td>0.20±0.13</td>
</tr>
<tr>
<td>Total protein</td>
<td>g/dl</td>
<td>7.4±0.8</td>
<td>8.2±0.7</td>
</tr>
<tr>
<td>BUN</td>
<td>mg/dl</td>
<td>12±4</td>
<td>13±4</td>
</tr>
<tr>
<td>Creatinine</td>
<td>mg/dl</td>
<td>1.3±0.3</td>
<td>1.6±0.3</td>
</tr>
<tr>
<td>CK</td>
<td>IU/L</td>
<td>238±196</td>
<td>230±246</td>
</tr>
<tr>
<td>ALP</td>
<td>IU/L</td>
<td>236±109</td>
<td>121±69</td>
</tr>
<tr>
<td>ALT</td>
<td>IU/L</td>
<td>8±5</td>
<td>7±7</td>
</tr>
<tr>
<td>AST</td>
<td>IU/L</td>
<td>22±10</td>
<td>22±12</td>
</tr>
</tbody>
</table>

\(^1\) Species 360, from: http://www.isis.org/support/MEDARKS/pages/Reference%20Ranges.aspx