Epidemiology and molecular biology of Elephant Endotheliotropic Herpesvirus 1 in the Asian elephant *Elephas maximus*

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Abstract

Herpesviruses are ubiquitous and are found worldwide, most animal species can be infected with multiple herpesviruses. Some cause clinical disease and others remain symptomatic throughout life. Herpesviruses are found in both captive and wild animals including Asian elephants (*Elephas maximus*).

Elephant Endotheliotropic Herpesvirus (EEHV) has been reported in both captive and wild Asian elephants, with a number of cases being reported in North America, Europe and Asia. It has been suggested that EEHV is associated with haemorrhagic disease, which has been attributed to a number of Asian elephant deaths, affecting mostly juveniles and calves. Clinical signs can vary from weight loss, lethargy, depression, cyanosis of the tongue and sudden death. Molecular testing using qPCR has enabled the detection of individual variants of EEHV, this thesis investigates the EEHV1 variant. EEHV1 has been highlighted as the variant that is more frequently associated with deaths.

This thesis includes five studies investigating different aspects of EEHV. Including, the relationship between pregnancy and EEHV viral shedding, the use of an amended human protocol for culturing endothelial cells, EEHV tissue tropism, a potential genetic or familial link between EEHV associated deaths and the detection of potential co-pathogens.

The main findings from this thesis include: 1) the use of a longitudinal study investigating a potential link between the physiological stress of pregnancy and EEHV viral shedding.

This study suggested there was no link between pregnancy and EEHV viral shedding however other stressors may be involved. 2) Using an amended human umbilical vein endothelial cell protocol, the culture of Asian elephant endothelial cells was successful. The cells from this study may be used in subsequent drug testing and vaccine development. 3) Quantitative PCR was used to determine EEHV1 tropism in tissues from two deaths associated with the virus. Tropism appeared to be for the heart and liver. 4) This thesis provides results from a preliminary study into a potential link between EEHV associated deaths. The data from an Asian elephant genogram shows there is the possibility of a genetic or familial link, which requires further investigation. 5) A number of tissues from deaths associated with EEHV and or death from other causes were investigated for the presence of potential co-pathogens, including the presence of encephalomyocarditis virus (EMCV), using microarray technology. The results indicated there were no co-pathogens present in the tissues.

This thesis adds to the current published data, and includes the first known preliminary study investigating a potential genetic link between elephant deaths due to EEHV.

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Declaration

Unless otherwise acknowledged the work presented in this thesis is original. No part has been submitted for another degree at The University of Nottingham or elsewhere. Any views expressed in the dissertation are those of the author.

Signed……

Date…….

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List of abbreviations

AIHV	Alcelaphine herpesvirus
ASPA	Non-Animal Scientific Procedures Act
AS	Array strip
AT	Array tube
BHV	Bovine Herpesvirus
BTV	Bluetongue virus
cDNA	Complementary DNA
CHV	Canine herpesvirus
CITES	The Convention on International trade in endangered species
CMV	Cytomegalovirus
CNT	Concentrative nucleoside transporter
CPE	Cytopathic effects
CPXV	Cowpox virus
Ct	Cycle threshold
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DJD	Degenerative Joint Disease
DRC	Democratic Republic of Congo
DTT	Dithiothreitol
EBV	Epstein-Barr virus
EEP	European Endangered Species Programme
EEHV	Elephant Endotheliotropic Herpesvirus
EGHV	Elephant Gammaherpesvirus
EHV	Equid herpes virus
EID	Emerging infectious disease
ELISA	Enzyme Linked Immunosorbent Assay
ELVIS	Enzyme linked virus inducible system
EMCV	Encephalomyocarditis virus

ENT	Equilibrative nucleoside transporter
FDA	Food and drug administration
GaHV	Gallid herpesvirus
gB	Glycoprotein B
gC	Glycoprotein C
gD	Glycoprotein D
HCMV	Human cytomegalovirus
HEC	Human elephant conflict
hEGF	human epidermal growth factor
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
HUVEC	Human umbilical vein endothelial cells
IFN	Interferon
IgG	Immunoglobulin G
ISIS	The International Species Information System
IUCN	The International Union for Conservation of Nature
kbp	Kilo Base Pairs
KS	Kaposi’s sarcoma
LAT	Latency associated transcript
LCMV	Lymphocytic choriomeningitis
LH	Luteinizing Hormone
LRRK	Leucine rich repeat kinase
MIT	Mouse inoculation test
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
NGS	Next generation sequencing
NSAIDS	Nonsteroidal anti-inflammatory drugs
OVHV2	Ovine Herpes Virus 2
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PCV	Porcine circovirus

PWMS	Post weaning multi-systemic wasting syndrome
qPCR	Quantitative Polymerase Chain Reaction
RFLP	Restriction fragment length polymorphism
RIF	Rifampicin
RNA	Ribonucleic acid
RT	Reverse transcription
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
SSC	Sodium citrate
SSP	Species Survival Plan
TAC	TaqMan array card
TB	Tuberculosis
TK	Thymidine kinase
TNF	Tumour necrosis factor
VZV	Varicella Zoster Virus
VWF	von Willebrand factor

Introduction

Elephant Endotheliotropic Herpesvirus (EEHV) is a host specific herpesvirus of both African and Asian elephants. It is thought that there are 7 variants of EEHV (EEHV 1-7); EEHV-2 and 6 found in African elephants and 1, 3, 4, 5 and 7 in Asian elephants (Latimer et al., 2011). Clinical disease varies between African and Asian elephants; in African elephants clinical disease is rarely reported. In Asian elephants by contrast, EEHV is the suspected agent of haemorrhagic fever syndrome. The progression of disease in this syndrome can be rapid and result in death within 1-5 days (Richman et al., 2000a). EEHV is present asymptomatically in most captive Asian elephants, and most animals go through periods of viral shedding without clinical disease. When clinical disease or death occurs, it is usually in juveniles aged between 1 and 5 years old. EEHV is not only present in captive populations, but also occurs in wild Asian elephants, deaths have been reported in India, Cambodia and Thailand, it has also been reported in wild elephants who can carry EEHV asymptomatically (Reid et al., 2006, Zachariah et al., 2013, Stanton et al., 2014).

In 2011 it was reported that there had been over 60 cases in the USA and 33 in Europe, with a fatality rate of 85% (Latimer et al., 2011), this has significant consequences for the

successful management of elephant populations in captivity. Asian elephants are currently listed as endangered on the International Union for Conservation of Nature (IUCN) red list, and are listed under the Convention on International Trade in Endangered Species (CITES) appendix II, in which international trading is regulated by the use of import and export permits (Choudhury et al., 2008).

EEHV can be studied in captive elephants to understand more about the virus, what if any impact this would have on wild populations is currently unknown. A greater understanding of how the virus is transmitted is needed, it is also unclear why some elephants can maintain a lifelong asymptomatic EEHV infection, whereas others develop clinical disease, and whether the genetics of the individual play a role in this. When clinical disease does occur, veterinarians usually treat the animal with anti-herpetic drugs such as famciclovir, however, it is unknown if other pathogens are also present with EEHV, and what effect this has on clinical disease.

Studying EEHV and understanding more about the virus and clinical disease could allow the development of new strategies to reduce the number of deaths in both captive and wild populations.

Chapter 1 – Literature review

1 Literature review

1.1 Taxonomy and distribution

The order Proboscidea contains the families Moeritherium and Deinotheriidae and sub order Elephantiformes, which contains elephants and their extinct relatives. Extinct Elephantiformes include Mammutidae (mastodons), Gomphotheriidae (Gomphotheres), and Stegodontidae (Stegodontids). The family Elephantidae includes the African elephant (*Loxodonta africana*), Asian elephant (*Elephas maximus*) and Mammoths (Figure 1). It has also been suggested that Elephantidae should be classified into two groups, Elephatini and Loxodontini. Elephantini would include the Asian elephant, mammoths and fossil relatives, and the second group Loxodontini would include African savannah and forest elephants and extinct relatives (Rohland et al., 2010).

The position of the Mammoths and their relation to African and Asian elephants has been the subject of much debate, with some studies suggesting mammoths are more closely related to African elephants and others suggesting Asian elephants. Using ancient mammoth mitochondrial DNA to investigate the evolution of Elephantidae, Krause et al. (2005) reported that the African elephant diverged from the mammoth first, and the Asian elephant diverged 440,000 years later, suggesting

that the mammoth is more closely related to the Asian elephant.

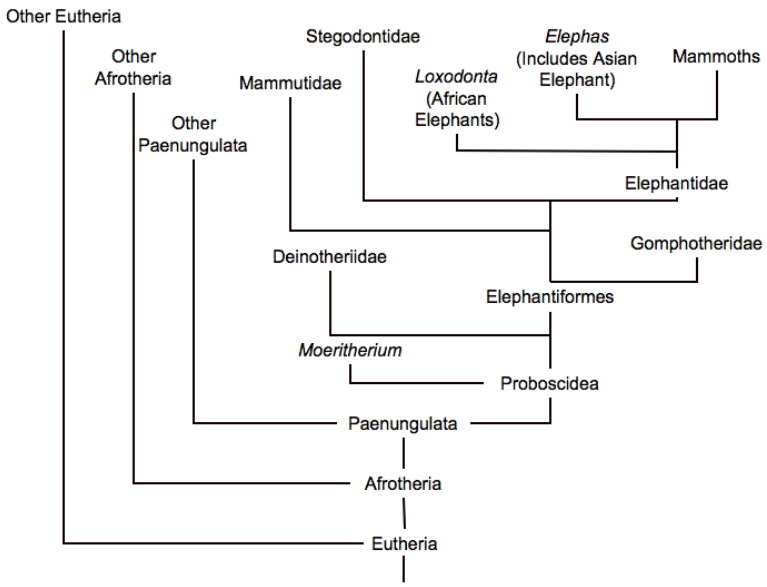

Figure 41: Cladogram showing the evolutionary position of the Asian elephant (Musgrove and Stockdale, 2011). The order Proboscidea contains the families Moeritherium and Deinotheriidae and sub order Elephantiformes containing elephants and their extinct relatives. Extinct Elephantiformes include Mammutidae, Gomphotheriidae and Stegodontidae. The family Elephantidae includes Loxodonta (African elephant), Elephas (Asian elephant) and Mammoths. Image reproduced with permission.

African elephants are found in 37 countries throughout Central and Southern Africa. They have become extinct in 3 countries, The Gambia (1913), Burundi (1970's) and Mauritania (1980's); they also became extinct in Swaziland in the 1920's but have since been reintroduced in the 1980's and 1990's (Blanc, 2008). It was reported in 2014 that African elephants had become extinct in Senegal, Somalia and Sudan (Larson, 2014, Al Jazeera News, 2014) (Figure 2).

There is much debate surrounding how many species of African elephant exist; currently the African species include the African bush elephant *Loxodonta* africana and the African forest elephant *L.africana cyclotis.* The forest elephant is found in forest environments; they are smaller in stature and possess straighter tusks in comparison to the bush elephant (Blanc, 2007).

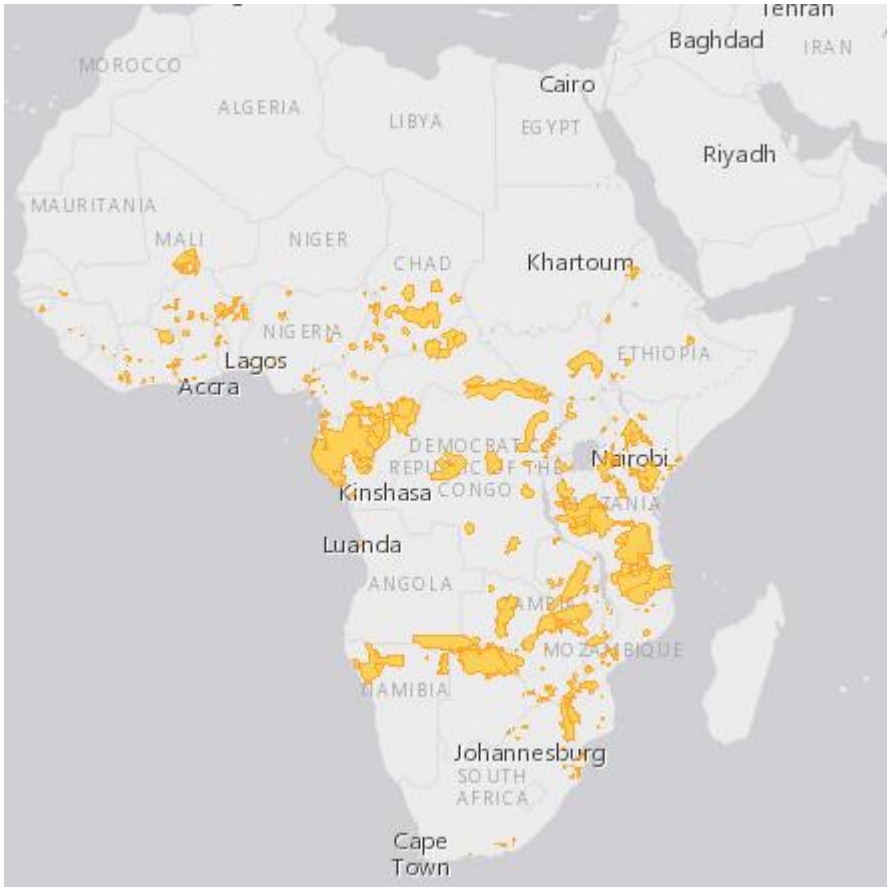

Figure 42: Range of the African elephant according to the IUCN red list. The current geographic distribution of the African elephant can be seen in yellow outlined in orange (International Union for Conservation of Nature, 2008). Image reproduced with permission.

1.1.1 Conservation concerns

African elephants (*Loxodonta africana* and *Loxodonta cylotis* combined) are currently listed as vulnerable according to the IUCN; between 1996 and 2004 they were listed as endangered, and since 2004 they have been listed as vulnerable. For a species to be classified as endangered it is classed as not critically endangered, but at a very high risk of extinction in the near future. Whereas for a species to be classed as vulnerable the species is facing a high risk of extinction but in the medium term future (Altshuler, 2006).

The most important reason for the decline of the African elephant is habitat loss; however, there are also other factors such as poaching and human elephant conflict. Illegal poaching has increased the value of ivory, male African elephants are targeted especially those with the largest tusks. This may have led to a shift in sex ratio with a reduction in the number of breeding males (Sukumar, 2006). Ivory poaching was a significant problem in the 1970's and 1980's; during this period around 100,000 elephants were killed each year. This led to a trade ban by CITES in 1989 (World Wildlife Fund, Unknown). The trade in ivory was restricted and generally pieces that can be dated to pre 1989 can be sold. In certain countries governments have implemented stricter rules, for

example, in Denmark, only ivory pre-dating 1947 only can be sold. If you inherit or own ivory you must be able to prove its age (Kennedy, 2011). Despite this ban, ivory poaching has still continued, with unregulated markets in 10 out of 37 African elephant range countries (Lemieux and Clarke, 2009). The success of the ivory ban has been under much debate as there have been variable results regionally. There were 65,000 elephants in Kenya in 1979, 19,000 in 1989 and 31,363 in 2007, which is an increase of 12,636 animals after the ban. By contrast, in 1979 there were 377,700 elephants in DRC, 85,000 in 1989 and 23,714 in 2007, this is a loss of 61,286 after the ban (Van Aarde and Jackson, 2006, Blanc, 2007).

1.1.2 Asian elephant

The Asian elephant (*Elephas maximus*) includes a number of subspecies: the Indian elephant (*Elephas maximus indicus*), the Sri Lankan elephant (*Elephas maximus maximus*), the Sumatran elephant (*Elephas maximus sumatranus*) and the pygmy elephant (*Elephas maximus borneenisis*) (Sukumar, 2006).

The range of the Asian elephant covers 13 countries in Asia (Figure 3). There are several dense populations located in Southern and North East India, as well as populations in

Myanmar (Burma), Malaysia, Sri Lanka, Nepal, Indonesia and Thailand (Sukumar, 2006) (Figure 3). Recent research using mtDNA and microsatellites supports the hypothesis that pygmy elephants located in Borneo are not from later importations, but are actually native to Borneo and have undergone an independent evolution from animals originally located there (Fernando et al., 2003).

A large number of Asian elephants are kept captive in range countries in a non-zoo environment. The use of elephants in agriculture and logging has been beneficial, due to their ability to move heavy loads (de Silva, 2013). Elephants can be of great economic value as there is a growing interest in ecotourism and tourist activities, such as elephant rides, trekking and elephant painting. Animals are housed in elephant camps along with their mahouts, who ride and take care of them. Asian elephants are also used for religious and cultural activities, especially in temple processions (Food and Agriculture Organization of the United Nations, 2002).

Figure 43: Range of the Asian elephant according to the IUCN red list. The location of Asian elephant populations are indicated by yellow areas (McCue et al., 2012). Image reproduced with permission.

1.1.2.1 Conservation concerns

According to the IUCN Asian elephants have been listed as 'endangered' since 1986, prior to that date, they were classed as very rare but thought to be stable or increasing. They have been listed under CITES Appendix I since 1975 (Delecluse and Hammerschmidt, 1993).

Currently the population is thought to be decreasing; a survey from 2006 estimated that there are between 38,500-52,500 elephants in Asia. The highest population density occurs in

India, home to 60% of wild Asian elephants (Sukumar, 2006). A number of Asian elephants are also kept in captivity worldwide, with 64 zoos in Europe and 36 zoos in the USA holding Asian elephants; this is 44% and 25% of the total number of zoos worldwide holding all elephants respectively (International Species Information System (ISIS), 2013).

There are a number of reasons for the decline of the Asian elephant including warfare, habitat loss, human elephant conflict and animals being taken from the wild to supply captive populations in zoos and circuses. Warfare has affected Asian elephants as there are a large number of unexploded landmines in countries such as Sri Lanka and Cambodia (Harris, 2000, Fernando et al., 2011). In Sri Lanka, approximately 3% of Asian elephant deaths occur from stepping on landmines each year; crippling injuries can also occur, including limb and trunk loss (Haturusinghe and Weerakoon, 2012).

Human elephant conflict (HEC) has also resulted in the loss of Asian elephants; this is mostly due to crop raiding, where the loss of elephants is the result of retaliation from farmers such as shooting, poisoning or electrocution. It has been estimated that over 200 elephants each year are killed in Sri Lanka due

to HEC (Fernando et al., 2011). Methods used by farmers to keep elephants away from their crops can be harmful to the elephants. This includes the use of 'Hakkapatas'', small land mines which the farmer covers in fruit or vegetables; when the elephant attempts to eat the food, the mine explodes, and may cause substantial damage to the head (Fernando et al., 2011).

In captivity Asian elephant populations are currently unsustainable, with more elephants dying than are being born. This may be due to high calf mortality and a large number of juveniles dying before they are old enough to have reproduced. Without the development of successful breeding programmes or the introduction of wild caught animals into captive groups, populations of captive elephants will continue to decline (Sukumar 1992). A study in 1998 concluded there are more stillbirths, and juvenile deaths in captivity, than occur in elephants in facilities located in range countries. The study included elephants in 25 European Endangered Species Programme (EEP) and Survival Species Plan (SSP) zoos located in Europe and North America, the Pinnawela elephant orphanage in Sri Lanka, and elephants at the Tamilnadu forest department in India. The results suggested that in zoos, 25% of elephant calves were still born, compared to zero at

Pinnawela and 3% in the Tamilnadu forest department. Twenty-three percent of elephants in zoos died between the ages of 0 and 5; this was compared to Pinnawela (9%) and The Tamilnadu forest department (7%) (Taylor and Poole, 1998). However, a more recent study suggests higher calf mortality in wild populations. In a study of young semi wild captive Asian elephants aged between zero and five years, it was reported that 26.3% of juveniles died before the age of one year, with the most common causes of death being general weakness/mother agalactica (50%) and accidents (37.5%). Whereas in those aged one to four years, the main cause of death was accidents (54.1%), and 34% of those aged between four and five died due to disease. The main cause of death overall was accidents in both females (43.3%) and males (41.7%) (Mar et al., 2012).

1.2 Genetics

1.2.1 Current knowledge

Studying the genetics of both humans and animals can provide valuable information on disease, populations and parentage. The use of genetic screening in humans can provide valuable information on the susceptibility to certain diseases. Screening has been developed for a number of diseases including the Leucine-Rich Repeat Kinase 2 (LRRK2)

gene mutation which is linked to familial Parkinson's disease (Nichols et al., 2005), and the BRCA1 and 2 genes linked to breast cancer (Ford et al., 1998, Recchia et al., 1999). However, there are many ethical arguments against genetically predicting the susceptibility to disease, especially diseases for which no treatment is available. Being made aware of disease susceptibility may cause psychological harm (Huang et al., 2002a). It also raises a number of questions such as can genetic information be kept confidential, and do family members have rights or access to the information (Lapham et al., 1996). Genetics can also be used to gather information on populations; such as the determination of genetic bottlenecks and their use in detecting recent population declines (Beckmann and Soller, 1986) and measuring genetic differentiation between populations (Tanzi et al., 1983, Williams et al., 1990).

Another use of genetics is parentage genetic testing which is a useful tool especially in the animal breeding industry; thoroughbred parentage testing has been used in the horse breeding industry to ensure animals are pure bred (Tozaki et al., 2001, Lee and Cho, 2006, Choi et al., 2012). Parentage testing has also been conducted on numerous other species including Atlantic salmon (*Salmo salar*) and North American

Bison (*Bison bison*) (Schnabel et al., 2000, Rengmark et al., 2006).

There are a number of methods and techniques that can be used to determine parentage, including microsatellites. Microsatellites are sections of DNA where a sequence is repeated, it may be a repeat of two different base pairs (di), three base pairs (tri) or four base pairs (tetra) (Turnpenny and Ellard, 2011). They are used as genetic markers as they are highly polymorphic, have a high mutation rate and they can occur at numerous locations throughout the genome (Crooijmans et al., 1993, Schlötterer et al., 1998).

Microsatellites are useful in determining parentage and genetic diversity of elephant populations. A number of microsatellites have been identified in African elephants, including dinucleotide (Nyakaana and Arctander, 1998, Comstock et al., 2000, Kongrit et al., 2008) trinucleotide (Fernando et al., 2001), and tetranucleotide repeats (Fernando et al., 2001, Archie et al., 2003). However, a smaller number have been identified for use in studying Asian elephant genetics (Eggert et al., 2000, Fernando et al., 2001).

Analysis of microsatellite markers and mitochondrial genes have been used to determine genetic differences in both African and Asian elephants. A study by Fernando et al. (2000) presented the genetic differences between 118 Asian elephants and four captive African elephants. The Asian elephants were located in mainland Asia (North India, Bhutan, Vietnam and Laos) and Sri Lanka, whereas the African elephants were located in zoos in the USA. PCR was used to amplify 630 nucleotides of mitochondrial DNA, which includes part of the control region. The results showed a distinct genetic variation between African and Asian elephants, with 54 polymorphic sites, whereas within Asian elephant populations a total of 27 polymorphic sites were found. Two distinct haplotype assemblages were found in the populations, assemblage α consisting of 7 haplotypes (A-G) and assemblage β consisting of 10 haplotypes (H-Q). The highest nucleotide diversity was observed in Bhutan, the highest haplotype diversity was in Vietnam, and the genetic diversity was lowest in India. Haplotype E was the most frequent, as it was observed in Bhutan, India, Laos, Vietnam, and Sri Lanka; by contrast, haplotypes A and Q were observed less frequently, with both of these haplotypes observed in Vietnam and Laos. Two haplotypes (E and L) were observed in Sri Lanka and the mainland (North India, Bhutan, Vietnam and

Laos). A further study by Vidya et al. (2004) looked at mitochondrial DNA (mtDNA) and microsatellites of 224 free ranging Asian elephants located in India, 5 haplotypes were reported. This study found 5 haplotypes and all samples from Southern India fell into clade β as described by Fernando et al. (2000).

Both microsatellites and mtDNA have been used to determine genetic diversity in captive populations. In a study of both captive and wild born Asian elephants located in North America, 20 microsatellite loci were recorded for 201 elephants. Of these, 164 were wild born, and 74 of them had a confirmed country of origin; 37 were captive born. Seventeen of the 20 microsatellites used were derived from those used in African elephants (Lei et al., 2012). Two mtDNA lineages were observed; these were clades α and β, as described previously by Fernando et al. (2000). Haplotypes of both α and β clades were present in wild born animals from Thailand, Myanmar and India, whereas only β clade haplotypes were found in individuals from Malaysia and Sri Lanka. Both wild born and captive Asian elephants showed similar genetic diversity (Lei et al., 2012) (Table 1).

Table 1: Mitochondrial DNA (mtDNA) clades present in elephant populations in Asia.

Location	mtDNA Clades present	Reference
Thailand	ɑ and β	(Lei et al., 2012)
Myanmar	ɑ and β	(Lei et al., 2012)
India	ɑ and β	(Lei et al., 2012)
Malaysia	B	(Lei et al., 2012)
Sri Lanka	B	(Lei et al., 2012)
Southern India	B	(Vidya et al. (2004)

1.3 General biology

1.3.1 Behaviour and biology

Elephants are mega herbivores and consume large amounts of low nutritional value vegetation and plant material. They are mixed feeders, engaging in both grazing and browsing, and feed for between 12 and 15 hours per day; although they may feed for longer. Therefore, they have large ranges similar to other mega herbivores; it has been estimated that range sizes of Asian elephants are between 34-320km^2 per individual. This figure is a conservative estimate, and the size of feeding ranges may be determined by a number of factors (Sukumar, 2006). In comparison the estimates of ranges for African elephants vary considerably within the literature Owen-Smith (1988) reported ranges of cow herds between 240km-1800km in the Kruger National Park and Tsavo East National Park respectively. Bull herds in the Pongola Nation Park ranged between 40-61km and cow herds were between 17 and 36km,

this is considerably small than those reported by Owen-Smith (1988), the results of the study may not accurately reflect the actual size of the African elephant range as the national park area was small at 103km^2.

There are numerous difficulties and limitations in studies looking at elephant ranges, distribution and factors affecting distribution. There are a limited number of studies within the literature that discuss these aspects of Asian elephant behaviour, these studies use methods such as direct observations, dung appearance rates, comparing statistics and correlations. Whilst this may be useful it is important to consider the methodological limitations such as the possibility of variable detectability, selection bias and limited spatial scales (which lead to range size underestimation) (Varma, 2008, Kumar et al., 2010, Rood et al., 2010, Gaucherel et al., 2010, Jathanna et al., 2015, Koirala et al., 2016).

1.3.2 Social behaviour

Social structure has been better studied in African elephants than in Asian elephants. African elephant society begins with a primary unit of a mother, and her offspring. The joint family unit or 'kin group' consists of a number of related cows (adult females), sisters, mothers, daughters and young males (de Silva, 2013). Group sizes depend on a variety of factors; a

larger group provides the benefits of close social contact and protection from predation. However, with a large number of animals, foraging becomes less efficient, with food then becoming a limiting resource, especially during the dry season. African elephant family groupings usually consist of 5-10 individuals, with Asian elephants forming smaller groupings of 5 individuals (Sukumar, 2003).

Male Asian elephants remain with their mother in the family unit until puberty. At around 6 years of age, they start spending time away from social groups, and by the age of 9, they spend most of their time alone or in small infrequent bachelor herds (de Silva, 2013). In another study, bulls associated with family units for around 23% of their time, especially when a female was in oestrus (Sukumar, 1992).

Herd structure and socialisation differs between Asian and African elephants, Asian elephants have looser social bonds with other individuals. Asian elephants in the Uda Walawe National Park (in Sri Lanka) tend to spend more time with certain individuals than with others; this may be due to family bonds, food locations or other preferences. As the individuals within a herd change on a regular basis, it is not clear if there

is a matriarch within Asian elephant society (de Silva et al., 2011, de Silva, 2013).

1.3.3 Reproduction

The gestation period of an Asian elephant is approximately 22 months. Young are reared by their mother and other cows within the herd, and there is a distinct absence of paternal care (Sukumar, 2003). When an African adult female elephant gives birth, it is a community event, with other adult females tending to the mother attentively; as elephants are allomothers, other females in the group take part in infant care (Lyons, 2015). In African elephants during the first few years of life a calf is frequently guided and touched by other members of the herd to provide reassurance (Moss, 1975).

The duration of the receptive phase of oestrus in Asian elephants varies from between 2 to 8 days and there are no obvious changes in behaviour in the females. The length of the oestrus cycle is on average 16 weeks long; the luteal phase lasts around 10 weeks and the follicular phase lasts around 6 weeks. The follicular (non-luteal) phase marks the point of ovulation and male interest increases at this point (Hess et al., 1983). The elephant has two surges of luteinizing hormone (LH) during the follicular phase. The first surge is named

anovulatory LH, which occurs 2 weeks after progesterone concentration drops. As ovulation is not induced at this point, the second surge occurs between 19-22 days after the first surge, and is named ovulatory LH as after this surge ovulation occurs. A double surge in LH has been confirmed in both African and Asian elephants. (Kapustin et al., 1996, Brown et al., 1999).

1.3.3.1 Captive breeding

Asian elephant populations in captivity are not sustainable, with higher death rates than birth rates due to low fertility rates and high calf mortality. To determine this, Wiese (2000) analysed data from 698 Asian elephants from the North American Asian Elephant Studbook using population management software. To predict population growth, age specific mortality and fecundity were calculated and to ensure a best case scenario, "optimistic" values were used. Ages were estimated for those that were wild caught, however, it was determined that these estimations would not have altered the predications.

The number of captive elephant calves dying in Europe during their first year of life is between 2.3 and 3.4 times greater when compared to working elephants in Burma (Taylor and Poole, 1998). To determine Taylor and Poole (1998) circulated a questionnaire to 14 EEP zoos, 178 SSP members, 16 Indian

zoos, and 23 other establishments with captive elephants such as logging camps. The questionnaire asked questions regarding Asian elephant demography, herd details, husbandry, management routines, breeding records, enclosure size, diet, and training. Of the 46 western establishments 22% responded; data was not given for the number of Asian establishments that responded. However, the results from this study are limited as the data was gathered in 1996, therefore it may be useful to repeat this study to understand the current situation, as significant changes may have been made in the intervening period.

It has been suggested that there are three factors for Asian elephant mortality in zoos: 1) being transferred to a new zoo has been noted to decrease survival, 2) origin; elephants in zoos that were wild born showed better survival compared to captive born, and 3) how recent the animal entered the population (Taylor and Poole, 1998). In a study of Asian elephant reproduction, it was reported that reproductive rates were lower in Asian elephants in European zoos compared to their range country counterparts (Taylor and Poole, 1998). Reproductive success was limited in European zoos, with 50% (10/20) reporting 1 live young per mature female every 6-7 years, whereas the Pinnawela elephant orphanage reported 1

live young per mature female every 5 years (Taylor and Poole, 1998). There are a number of problems which may increase failure to conceive in captivity. Problems include limited opportunities for males to mate in captivity due to restricted access to females, this is also observed in range country captive Asian elephants (Taylor and Poole, 1998). Females in captivity are not able to choose their mating partner; this is not helped by a lack of males in captivity. There are difficulties in keeping male Asian elephants in captivity due to musth, where bulls may pose a danger to other elephants and keeping staff, during this time the male may be tethered or confined (Taylor and Poole, 1998). Due to the above reasons natural reproduction in captivity is difficult; successful artificial insemination may increase the chances of captive populations becoming more stable (Brown et al., 2004).

Zoo populations have previously been sustained by the import of wild animals, and in the past 40 years, growth in the captive population is due to importations. This study is limited as data was collected in 1999, however, a repeat of this study would give more up to date population predictions (Wiese, 2000). Increasing the success of breeding in captivity is considered important to sustain captive populations; because there are a limited number of successfully breeding males and

females in captivity, this will eventually lead to a reduction in the genetic pool (International Species Information System (ISIS), 2013). The population in North America is currently aging; this suggests that the number of fertile females will decrease further in this group; it is likely to be the same in Europe.

It may be possible that in the future, management changes may lead to increased fertility rates or a reduction in infant mortality rates.

1.3.4 Elephant immune system

The immune system protects the body from foreign bodies and microbes such as bacteria, parasites and viruses. There are two main types of immunity: acquired and innate. Acquired immunity is only present in vertebrates and is characterised by memory and specificity, there are two main types of cells which carry out the acquired immune response and "remember" the pathogen, B cells and T cells. (Akira et al., 2001, Alberts et al., 2002). B cells carry out an antibody response (humoral response), activated and antibodies are secreted. The antibodies then bind to the foreign antigen which then causes inactivation. The result of this is that the foreign antigen is not able to bind to the host (Alberts et al

2002). T cells are involved in a cellular response (cell mediated response), where T cells react to the presence of foreign antigens present on the cell surfaces of the host, the T cell is therefore able to "kill" a virus infected cell before it is able to replicate (Alberts et al 2002).

As B cells, T cells and NK cells are able to create immunological memory it leads to an enhanced immune response when exposed to a pathogen previously encountered (Orme, 1988, Kurtz and Franz, 2003). However, the acquired immune system may recognise harmless molecules as being harmful, this can result in conditions such as asthma or allergies (Hammad and Lambrecht, 2008, Holgate, 2012).

Innate immunity is evolutionarily older and found in many diverse organisms. It is characterised by the capability to distinguish between self and foreign "material" and the response is not specific to individual pathogens, however, unlike acquired immunity it is not able to provide long lasting immunity (Akira et al., 2001, Alberts et al., 2002). Acquired immunity is known to develop slowly, especially during initial exposure, during this time the body is protected from infection by the innate immune system (Alberts et al., 2002). There are a number of cells that function within the innate immune systems such as NK cells, macrophages and neutrophils. In the innate immune response NK cells are not directly involved

in destroying or attacking foreign microbes, but are able to destroy compromised host cells such as virus infected cells. Recent evidence suggests they are part of both the acquired immune system and the innate immune system, they have the ability to "remember" pathogens like B cells and T cells. NK cells share similarities with T cells, such as the ability to conduct perforin or granzyme mediated killing (DiSanto et al., 1995, Paust et al., 2010). Macrophages are able to bind bacteria molecules on their surface, the macrophage is then triggered and the bacteria is engulfed and destroyed through a process called respiratory burst (Janeway et al., 2005). Another cell that is able to destroy foreign microbes using respiratory burst is neutrophils, which are the most common type of phagocyte (Stvrtinova et al., 1995). Neutrophils have granules within their cytoplasm which are able to kill or inhibit growth of foreign microbes such as bacteria or fungi (Brinkmann et al., 2004).

There is limited information about the elephant immune system and this appears to be a gap in the literature. The morphology and function of the elephant immune system is thought to be the same as that in other mammals, with bone marrow being the main site for the maturation of B lymphocytes and the thymus for the maturation of T

lymphocytes (Lowenstine, 2006). Anatomically, the immune system of the elephant is also made up of lymph nodes (associated with lymphatics), hemal nodes (associated with the vascular system), tonsils, spleen, bone marrow and mucosal associated lymphoid tissue (in the form of palatine tonsils) (Lowenstine, 2006).

Studying the immune system of the Asian elephant may provide more information on the effectiveness of the immune response, disease susceptibility and general immune function. To investigate the immune system many studies in other species have looked at the expression of cytokines, which are produced by cells such as T lymphocytes, B lymphocytes, endothelial cells, macrophages and mast cells. The expression of cytokines has been studied due to their involvement in the hosts' immune response, sepsis, inflammation and as a marker of disease severity (Oberholzer et al., 2000, Bozza et al., 2007, Goldberg, 2009). There is limited information on cytokine expression in the Asian elephant. Landolfi et al. (2009) developed and validated a series of cytokine RT-PCR assays to characterise immune responses in Asian elephants. Cytokines investigated included TNF alpha (cell signalling protein), TGF beta (multifunctional), interferon gamma (induces an array of immune responses), interleukin 2

(regulates homeostasis and immune activation particularly of T cells), interleukin 4 (induces differentiation of helper T cells into TH2 cells), interleukin 10 (inhibits macrophage functions), and interleukin 12 (activates immune responses and may play a role in haematopoiesis), and could be detected in the peripheral whole blood obtained from Asian elephants, (Hennessey et al., 1993, Wolf et al., 1994, Rincón et al., 1997, Thompson et al., 1998, Aggarwal, 2003, Gaffen and Liu, 2004, Gattoni et al., 2006).

Landolfi et al. (2009) has contributed a number of cytokine gene sequences for future study. It may also serve as the foundation for a number of future studies including comparing the immune responses of both healthy and diseased individuals, this may allow for a greater understanding of disease susceptibility. However, the functional significance of these key cytokines in Asian elephants must be inferred from other species, as there is no direct evidence confirming their function in the Asian elephant.

RT-PCR studies of cytokine gene expression require only the gene sequence for the gene of interest and as such are relatively easily transferred to exotic species. In contrast, the use of functional assays (for example ELISPOT assays to

measure T cell activity) require reagents that are more difficult to generate, for example, specific antibodies and may require extensive optimisation of culture systems to study elephant immune cells in vitro. It is also difficult to perform experimental studies in live animals and there are a number of ethical concerns especially when working with endangered species.

1.3.4.1 Immunomodulation and stress

Immunomodulation is the normal processes by which the immune system regulates itself. Regulatory T cells are a type of T cell that are modulate and supress the immune response and so maintaining homeostasis. It has been shown that regulatory T cells are able to inhibit both cytokine function and T cell proliferation and downregulation, it is also though that they may play a role in preventing autoimmunity (O'garra and Vieira, 2004, Kondelkova et al., 2010). There are a number of proposed suppression mechanisms used by regulatory T cells, such as the prevention of co-stimulation, the production of inhibitory cytokines such as interleukin 35 and 10 and the production of Granzyme B which is able to induce effector cell apoptosis (Read et al., 2000, Gondek et al., 2005, Collison et al., 2007, Walker and Sansom, 2011). If dysregulation of regulatory T cells occurs it may lead to the development of certain autoimmune diseases (Kondelkova et al., 2010).

Certain medications (immunomodulators) can be used to normalise or regulate the immune system which are used in the management of certain disease such as Crohn's disease and inflammatory bowel disease (Punati et al., 2008, Sokol et al., 2010). There are other types of immunomodulators such as female sex hormones which alter the sensitivity of the immune system (Wira et al., 2015).

Psychological stressors can have multiple effects on the immune system, impairing its ability to fight off infection and alter normal immune response, such as the disruption of certain networks of communication for instance signals linking the endocrine system, the nervous system and the immune system and the inhibition of antibody production (Rabin, 1999). The sympathetic nervous system can be continuously activated under chronic stress, when the parasympathetic nervous system does not counteract, this the immune system can be activated, this is achieved through an increase in proinflammatory cytokines, having been observed in patients with depression (Won and Kim, 2016).

1.4 Diseases of elephants

1.4.1 Non-infectious diseases

There are a number of non-infectious diseases and conditions affecting elephants. This review will focus on several important

non-infectious conditions of captive elephants including: obesity, foot problems and degenerative joint disease. However, the list is not exhaustive.

1.4.1.1 Obesity

It has been suggested that obesity is a problem in both captive Asian and African elephants; this is the presence of excessive amounts of body fat. One study suggested that captive Asian elephants were 21% heavier than their wild counterparts, and African elephants were 27% heavier (Palanivelrajan et al., 2015). There are a number of suggested reasons as to why obesity is occurring including: 1) hay quality, this is a staple part of the captive elephant diet, but it may not provide the correct proteins, minerals and vitamins in comparison to a wild elephant diet, 2) overfeeding, 3) low fibre diet and 4) insufficient exercise/activity (Hatt and Clauss, 2006, Association of Zoos and Aquariums, 2011a). Captive elephant diets differ to their wild counterparts as they are fed pellets, which provide vitamins and minerals not found in hay. They are also fed a number of supplements including biotin, vitamin E, Zinc, bran, oats and produce (Bowling et al., 1997). Produce includes fruits and vegetables such as apple, carrots and bananas, as they are considered to be high in sugar this may be a contributing factor in obesity (Coote and Bruford, 1996). A number of publications have suggested that a lack of

exercise may lead to increased health problems in captive elephants, such as pododermatitis, degenerative bone disease and obesity (Mikota et al., 1994, Roocroft and Oosterhuis, 2001, Gaskin et al., 1980). Obesity can also lead to other conditions such as foot lesions and joint problems (Csuti et al., 2001). It is thought obesity in captive elephants may be contributing to their low reproductive success. As obesity may also be linked to the presence of benign uterine tumours (leiomyomas) leading to infertility, as is thought to be the case in humans (Hildebrandt and Göritz, 1995, Montali et al., 1997, Sato et al., 1998).

In the USA AZA provides zoos with recommendations for exercise programmes to try and increase the amount of activity captive elephants undertake (Association of Zoos and Aquariums, 2011a). Wild elephants are known to undertake large amounts of walking each day; Leggett (2010) found that wild African elephants travelled between 0.38-0.63km/hr, captive African elephants have been found to travel around 0.4km/hr which is towards the lower end of that travelled by their wild counterparts (Leighty et al., 2009, Rothwell et al., 2011). However, these studies only included a small number of elephants, the size of the enclosure may influence the ability or desire to exercise. It is also important to consider

exercise is not only about the distance walked, but also other activities engaged in during that time, such as varied terrain, pulling or pushing trees which would involve muscular effort. More research is needed to better understand activity and space used by captive elephants.

1.4.1.2 Foot problems

Foot problems are commonly seen in elephants, 50% of which will be affected by a foot problem at least once in their lifetime (Csuti et al., 2001). With captive elephants, enclosure design is important and has an impact on foot heath; it is essential to provide the correct floor substrate and drainage in both elephant barns and outdoor areas. In outdoor areas, limestone, granite or clay soil should not be provided as it may become lodged into crevices on the soles of the feet. Foot health can be improved by providing an enclosure that is similar to their natural habitat including natural substrates and suitable vegetation (Buckley, 2008). There are a large number of non-infectious and infectious conditions that can affect elephant feet. Non-infectious conditions include foreign bodies, irritation and abrasions. Infectious diseases include foot and mouth disease, which can lead to lameness due to swollen skin around the toenails; elephant pox which can lead to the feet becoming infected and toenails can detach from the foot; and pododermatitis which may appear as a localised abscess

or infection around the nails, this may be due to moisture and the lack of routine foot care (Murray, 2006). Foot problems and injuries especially lacerations and foreign bodies also occur in wild populations and are thought to be common (Csuti et al., 2001).

1.4.1.3 Degenerative joint disease (DJD)

DJD is a common problem in captive Asian and Africa elephants and is associated with lameness. There is no single cause for DJD and other joint problems such as arthritis, but contributing factors may include: using a hard concrete substrate, inadequate exercise, obesity and wet conditions (Fowler and Mikota, 2006). The chaining of elephants may also contribute to the development of DJD, as they maybe chained for long periods and their movement is limited. Elephants that resist chaining or pulling on the chain may cause more joint damage (Firyal and Naureen, 2007). It is also thought that DJD may develop from the acquisition of infectious agents such as *Mycoplasma*. A previous study looking for arthritogenic agents in two groups of captive elephants (62 Asian elephants and 5 African) found 28/35 genital tracts were colonized by one or more *Mycoplasma spp*. Both rheumatic disorders and lameness were found in some animals, and were found to be correlated with *Mycoplasma* antibody titres (Clark et al., 1980).

The most important clinical sign is lameness. However, the presence of lameness occurs after structural changes have occurred to the joint. Cartilage damage within the joint leads to proteoglycans being released into the synovial fluid. The result of this is an inflammatory reaction leading to inflammation of the synovial membrane (synovitis). Further inflammation of the joint is then caused by a destructive release of enzymes mediated by prostaglandins and interleukins (Fowler and Mikota, 2006). As the disease progresses fibrosis of soft tissue and inflammation can cause a decrease in movement, and advanced DJD can lead to bone pain (Fowler and Mikota, 2006).

A diagnosis can be made with the use of radiography. Most captive elephants are trained to lift their feet for inspection, this enables radiography without the use of a chemical restraint (Hittmair and Vielgrader, 2000). Radiography can show characteristic changes associated with DJD such as sclerosis and decreased joint space (Fowler and Mikota, 2006).

Treatment of DJD can include changes in husbandry and a variety of different treatments including nonsteroidal anti-inflammatories (NSAIDS). NSAIDS are used as they interrupt

prostaglandin synthesis, however overuse may also supress proteoglycan synthesis, proteoglycans are an important constituent of cartilage (Fowler and Mikota, 2006). Successful treatment can include glucocorticoids, antibiotics and hot/cold compresses (Palanivelrajan et al., 2015). A number of enrichment opportunities available to captive elephants may help to maintain joint health. These include substrates that allow for digging and swimming, which relieves joints as it provides a non-weight bearing exercise. However, exercise should not begin until inflammation has been reduced (Fowler and Mikota, 2006).

1.4.2 Infectious diseases

1.4.2.1 Mycobacterium spp

Mycobacterium is a genus of Actinobacteria, this genus includes many species found in the environment, and it also contains pathogens known to cause diseases in both humans and animals such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*. This review concentrates on pathogens of this genus known to cause the disease tuberculosis.

Mycobacterium tuberculosis is an obligate bacterial pathogen, and is one of the causative agents of tuberculosis (TB). It can be difficult to identify the bacteria using gram staining as

there is a waxy coat on the cell surface making it impervious to the staining technique, this means it can appear as both gram negative and gram positive in clinical tests (Fu and Fu-Liu, 2002).

TB was described in elephants by ancient people more than 2000 years ago (Iyer, 1937). Two circus elephants died of the disease in 1996 (Mikota et al., 2001). Symptoms of TB in elephants include weight loss, weakness and coughing, although it can go undetected in elephants for many years and may not be noticed until post mortem examination.

A number of tests have been used to diagnose the disease in elephants including the intradermal tuberculin test, enzyme linked immunosorbent assay (ELISA) and acid-fast smears (The United States Animal Health Association Elephant tuberculosis subcommittee, 2012). The Tuberculin skin test called the Mantoux skin test is used to diagnose the disease in humans, this involves injecting a standard dose of tuberculin intradermally, if a person has been exposed, an immune response occurs and the reaction is read by measuring the size of the papule. Elephants can also be diagnosed using a skin test, although tests may be altered depending on the species being tested; different tuberculin extracts may be

used, standard tests in the UK for cattle use a preparation of *M.bovis* AN5 (Mikota et al., 2001). Intradermal tuberculin tests are also used to diagnose TB in other animals such as cattle. One study of captive elephants in North America surveyed 539 elephants of which 18 tested positive (3.3%) using the intradermal tuberculin test (Mikota et al., 2000). A more recent study used The Elephant TB STAT-PAK rapid flow test which detects antibodies in sera specific to *M. tuberculosis*, and bacterial cultures from trunk washes. The small study of four elephants located in Thailand reported the presence of *M. tuberculosis* in the four animals tested. The study also concluded transmission *of M. tuberculosis* to the four elephants, was likely to have been contracted from humans (Angkawanish et al., 2010).

The United States Animal Health Association has published guidelines on the testing and treatment for TB. The guidelines state that all captive elephants must be tested annually using the rapid flow Elephant TB STAT-PAK antibody assay, using a blood sample (Mikota et al., 2000, Mikota et al., 2001).

There are three management options when TB occurs in elephants, these are; isolation, euthanasia and treatment. It is thought that the most effective therapy depends on rapid

diagnosis, and includes adequate plasma drug levels, adequate length of treatment and close monitoring of the elephant. It is suggested that antibiotics are administered orally but directly, as it is not recommended to put the drug over the feed which likely reduces the efficacy. Drugs used for initial treatment include; isoniazid, pyrazinamide, rifampicin, ethambutol and streptomycin. There is currently no published data indicating the cost of treating TB in elephants.

Multi drug resistance, is thought to occur; typically resistance to isoniazid and rifampicin (Törün et al., 2005), if this occurs, it is recommend to use second line drugs. Second line drugs include aminoglycosides (amikacin and capreomycin), ethionamide, and quinolones (Jordheim et al., 2013). Although it can be difficult to detect TB in any species, early diagnosis and treatment are thought to be necessary. In humans there are several factors that influence the critical intervention point where treatment is initiated including: age, access to medical services, and the presence of co-infections (Tsara et al., 2009).

As described above TB can be difficult to detect and also difficult to treat. Nepal has taken action to try and reduce the number of cases of TB in Asian elephants and is a high priority

issue for the Nepalese government, which began elephant TB surveillance in 2006, followed by the standardisation of TB treatment in 2008. The Nepalese government aimed to minimize the transmission of TB at the captive-wild interface with the introduction of the Nepal Elephant Tuberculosis Control and Management Action Plan. The aims of the plan included introducing a programme to diagnose and treat TB among both captive elephants and their mahouts, establish a monitoring system, develop and establish an education programme to teach elephant staff, tourists and local communities and to also include control initiatives for TB in humans and livestock including a collaborative One Health initiative. However, the plan does not detail how the success of the programme is going to be measured or how the data is going to be reported (Department of National Parks and Wildlife Conservation, 2011). There is no published data to show if this control and management action plan was successful. Assessing the success of the control measures requires further research, and if the measures were shown to be successful, they could be introduced into other range countries.

1.4.2.2 Bacillus anthracis

Bacillus anthracis, is a gram positive, rod shaped, endospore forming bacteria (Baillie and Read, 2001). It is the causative

agent of anthrax, is highly contagious and has been reported in almost all mammalian species. Anthrax can be cutaneous, where lesions appear on exposed skin, or pulmonary or gastrointestinal with clinical signs including anorexia and fever. Gastrointestinal anthrax can cause cyanosis, dyspnoea and sudden death. A combination of anti-inflammatories, antibiotics and analgesics have been used successfully to treat animals (World Organisation for Animal Health et al., 2008).

Elephants can become infected through eating contaminated vegetation and soil, and infection has been reported in both captive and wild populations of African and Asian elephants (Rao and Acharjyo, 2006). In 1927 an outbreak of anthrax among staff and elephants at a zoo in the UK was reported, the study does not indicate if the elephants were African or Asian. The outbreak resulted in the sudden deaths of two elephants (clinical signs were not reported). Blood smears were taken from the heart, liver, kidney, spleen and lungs, and tissues were also submitted for aerobic and anaerobic cultures. Large, square ended bacilli were visible in all smears. A second elephant died and was subjected to a post mortem examination, multiple smears also showed numerous bacilli. (Scott, 1927). A diagnosis of anthrax was made for both animals. The source of the infection remains unclear (Scott,

1927). A second outbreak of anthrax was reported at Chester Zoo, UK in 1964 this included African elephants (Jordan, 1964).

A number of deaths have occurred in wild populations; clinical signs were not observed; however, post mortem examinations have been conducted on a number of animals. Examination of the carcass showed blood leaking from the eyes and trunk, post mortem examinations revealed absence of rigor mortis, dark tarry blood and uncoagulated blood (Priya et al., 2009, Yasothai and Shamsudeen, 2014).

Priya et al. (2009) reported blood smears from trunk and ocular blood samples from a wild Asian elephant revealed a number of truncated-end rods typical of *B. anthracis* infection. Anthrax was confirmed using a mouse inoculation test (MIT), where infected tissue from the animal was inoculated into mice, which were then killed and examined for the presence of *B. anthracis* (Priya et al., 2009).

Yasothai and Shamsudeen (2014) confirmed the presence of anthrax in an Asian elephant using blood smears. The smears displayed the characteristics of the McFadyean reaction, which is a staining reaction that displays a blue cell surrounded by a

pink capsule, characteristic of an anthrax bacilli infection (Yasothai 2013). Anthrax has also been discovered to be the cause of death retrospectively in a number of cases; a study in the Etosha National park, Namibia reported 10 Anthrax related deaths in wild African elephants between 1969 and 1970 (Ebedes, 1976).

1.4.2.3 Cowpox virus

There are a number of zoonotic pox viruses including cowpox virus (CPXV) which is a member of the Orthopoxvirus genus within the *Poxviridae* family. CPXV is thought to be the causative agent of the disease cowpox, similar to the highly contagious and now eradicated smallpox in humans. CPXV inspired the small pox vaccine developed by Edward Jenner. Human cases are rare, though a case in a laboratory worker has been reported. An ulcerated lesion appeared on the finger of the employee, other symptoms included chills, headaches and body aches. The employee had handled mice that were inoculated, the inoculant was discovered to have been contaminated with CPXV (Robert, 2012).

CPXV is frequently transmitted by rodents, especially woodland rodents, such as the common rat (*Rattus norvegicus*); one report indicated that 20% of wood mice, 20% of bank voles (*Myodes glareolus*) and 33% of field voles

(*Microtus agrestis*) had antibodies to cowpox virus (Crouch et al., 1995).

CPXV has occasionally been transmitted to zoo animals; this includes captive Asian elephants (*E. maximus*), anteaters (*Myrmecophaga tridactyla*), and okapis (*Okapia johnstoni*). This also includes a number of felid species such as the lion (*Panthera leo*), puma (*Puma concolor*), jaguar (*Panthera onca*), and cheetah (*Acinonyx jubatus*). There have been reports of transmission from elephants to humans. Kurth et al. (2008) and Hemmer et al. (2010) described CPXV transmission from rats to Asian elephant and subsequent transmission from elephant to humans. CPXV is also thought to have been responsible for some stillbirths in Asian elephants. An active CPXV infection was detected in a stillbirth from a previously vaccinated dam. Signs of cowpox infection seen in the calf included papules over the skin, as well as lesions on the trunk and around the eyes (Wisser et al., 2001).

1.4.2.4 Leptospira spp

Species of the *Leptospira* genus are spirochaete bacteria; the genus includes some pathogenic species including *L. interrogans,* the causative agent of Leptospirosis.

Leptospirosis is a common rodent borne zoonotic infection. In a study of wild brown rats (*Rattus norvegicus*) in the UK, using multiple testing methods including serological and non-serological testing, 14% of rats were positive for *Leptospira spp* by at least one test (Webster et al., 1995). In contrast a more recent study of *R. norvegicus* in Denmark found a prevalence rate of between 48 and 89% in five different sewer locations (Krøjgaard et al., 2009). *Leptospira spp* are also found in livestock and domesticated animals. Most cases of leptospirosis in livestock are sub clinical, but where an acute infection is present reproductive loss may occur through: stillbirth, abortion, and infertility (Bolin and Alt, 1999). Infected bovine calves may show clinical signs such as jaundice, pulmonary congestion, fever, meningitis and death may also occur (Bolin and Alt, 1999). In cattle younger animals are more seriously affected in comparison to older animals (Bolin and Alt, 1999). Leptospirosis can be diagnosed using various methods including serological testing. Non-serological testing methods such as PCR can also be used (Van Eys et al., 1989). A more recent study used nested PCR to confirm the presence of leptospires in the urine of cattle (Daibata et al., 1999).

Leptospirosis has been reported in captive Asian elephants; in a study of 42 captive Asian elephants in India, all were found to be positive for at least one *Leptospira interrogans* serovar (Vengadabady et al., 2009). By contrast, another study in India found a lower prevalence rate, 7/51 (13.7%) serum samples tested positive for the presence of *L.interrogans* serovar antibodies (Shivraj et al., 2009). Death due to Leptospira infection in Asian elephants has been reported, including the death of a 6 year old juvenile in India; nephritis and adrenal cortical infection were observed in this case (Fowler and Mikota, 2006).

In elephants, clinical signs may include weight loss, anorexia, uveitis, jaundice, ventral oedema, genital and skin lesions. Treatment with tetracycline, doxycycline and enrofloxacin have been successful (Fowler and Mikota, 2006).

1.4.2.5 Yersinia spp

Yersinia is a genus of gram negative, rod shaped bacteria that is capable of both aerobic and anaerobic respiration. A number of pathogens within the *Yersinia* genus are pathogenic to both humans and animals, including *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*. *Y. pestis* known to cause plague, evolved from the relatively benign *Y. pseudotuberculosis* an estimated 1500-20,000 years ago

(Eisen and Gage, 2009). *Y. pestis* is primarily flea borne and a large number of flea species are able to be infected (>80). Despite the diverse number of mammalian hosts, many mammals are not able to develop a high level infection required to infect feeding fleas (Eisen and Gage, 2009). Highly susceptible species include rodents. With a high abundance of rodents, there is often a rapid and sustained spread which can elevate both human and wildlife exposure (Eisen and Gage, 2009). In Europe a number of rodents are thought to be maintenance hosts, whereas in North America this includes black-tailed prairie dogs. A previous study investigated the prevalence of *Y. pestis* in the black-tailed prairie dog eco-system using nested PCR (Hanson et al., 2007). Hanson et al. (2007) found in 2003, 23% of burrows contained fleas positive for *Y. pestis*. A PCR test for the detection of *Y. pestis* was developed in 1994 (Norkina et al., 1994), more recently a real-time PCR has been developed to detect *Y. pestis* in the sputum of humans (Loïez et al., 2003). The development of antibiotics led to a drop in the number of plague cases; initially treatment was with sulfonamide and then streptomycin (Galimand et al., 2006). Currently streptomycin remains the antibiotic recommended for treatment, however, multidrug resistance has been reported (Galimand et al., 1997, Galimand et al., 2006).

Exposure to *Y. pestis* has been reported in a wild African elephant, however, there have been no reports in Asian elephants. A serological study in South Africa found 1/330 (0.3%) of wild African elephants were seropositive (Gordon et al., 1979). Infection and clinical disease have not been described in elephants and little is known about the epizootic potential.

1.4.2.6 Toxoplasma gondii

Toxoplasma gondii is a ubiquitous obligate, intracellular parasite found in a large number of warm blooded mammals, and is the causative agent of Toxoplasmosis (Centers for Disease Control, 2015). *T. gondii* undergoes sexual reproduction within its definitive hosts, which are thought to be Felid species, including domestic cats (Webster, 2007).

T. gondii infection has been reported in both wild and captive populations of elephants with no apparent clinical signs (Dangolla et al., 2006). A number of previous studies have investigated the prevalence of *T. gondii* in both captive and wild elephant populations. Using a modified agglutination test, Dangolla et al. (2006) found a serologic prevalence of (14/45) 32% in captive Asian elephants located in Sri Lanka. By contrast, a more recent study conducted in Thailand used a latex agglutination test to detect specific IgG antibodies, and

determined that antibodies were present in 33.04% of those studied (Wiengcharoen et al. (2012). The route of transmission to elephants and other herbivores is thought to be by ingesting oocysts shed by felids by faecal-oral transmission (Dubey, 2006), in contrast to the ingestion of infected meat associated with infection in carnivorous species (Dubey and Beattie, 1988).

1.4.2.7 Encephalomyocarditis virus (EMCV)

EMCV is a single stranded RNA virus with a worldwide distribution; it is a small non-enveloped *Cardiovirus* of the subfamily *Picornaviridae* (Grobler et al., 1995). It is thought to cause encephalitis, myocarditis, reproductive disorders and sudden death in pigs (Dea et al., 1991, Koenen et al., 1999). There are currently no drugs available for the treatment of EMCV, however, treatment with iodine-based disinfectants at 60^{o}C for 30 minutes can inactivate the virus (Carocci and Bakkali-Kassimi, 2012).

The route of transmission is thought to be either from eating infected rodents (in carnivorous species), or from food and water supplies contaminated with the faeces of infected rodents (Maclachlan and Dubovi, 2011). EMCV has been reported worldwide in a number of species, including in zoos located in Australia (ring tailed lemur (*Lemur cata*), squirrel

monkey (*Saimiri sciureus*), mandrill (*Mandrillis sphinx*), chimpanzee (*Pan troglodytes*), pygmy hippopotamus (*Choeropsis liberiensis*), and goodfellow's tree kangaroo (*Dendrolagus goodfellowi*)) and Italy (Black lemur (*Eulemur macaco macaco*), ring tailed lemur (*Lemur cata*), red ruffed lemur (*Varecia variegata*), white fronted lemurs (*Eulemur albifrons*), Barbary macaque (*Macaca jacchus*), common marmoset (*Callithrix jacchus*)) (Reddacliff et al., 1997, Canelli et al., 2010).

EMCV has also been reported in wild African elephants in South Africa, and in pig herds in Belgium, Cyprus, Greece, UK, Austria, USA, Korea and Japan.

A study of European (Italy, Greece, Belgium, Cyprus, France, Austria and UK) pigs using serologic detection, suggested prevalence rates in endemic areas (defined as an area where EMCV occurs regularly) of 8.2% to 66% and non-endemic areas of 0% to 5.4% (Maurice et al., 2005). Serological prevalence rates in pigs have also been reported outside Europe including Korea (6.9-12.4%) and Japan (up to 56%) (Sato et al., 1998, Lyons, 2015).

EMCV has been reported in both wild and captive populations of African and Asian elephants, and has resulted in death (Simpson et al., 1977, Gaskin et al., 1980, Grobler et al.,

1995, Richman et al., 1999, Lamglait et al., 2015). A variety of tissues can test positive for EMCV using methods which detecting responses from the host's immune system, and those directly detecting EMCV RNA. Viral isolation has been successful from a number of tissues including heart, liver, kidney, spleen and pancreas in a range of primates and hoofed animals (Wells et al., 1989). Osorio et al. (1996) tested an EMCV vaccine in pigs, baboons and macaques; the vaccine was developed using a genetically modified mengovirus. In baboons, it may be necessary to give multiple vaccinations, depending on the size of the animal and the dosage administered. An alum adjuvant vaccine for use in pigs has also been developed, this vaccine uses an inorganic aluminium salt as the adjuvant; trials of the vaccine protected against EMCV challenge in pregnant sows. The number of abortions and stillborn animals was less in the vaccinated animals compared to controls (Jeoung et al., 2012). There have been a number of vaccine trials, but a vaccine for EMCV in pigs is not currently available for commercial use in the UK (National Office of Animal Health, Unknown) or in the USA (United Sates Department of Agriculture and Animal and Plant Health Inspection Service, 2013). An experimental oil-adjuvant vaccine has also been trialled in a group of juvenile African elephants after it showed success in both mice and

pigs. The juvenile elephants were vaccinated and subsequently exposed to EMCV; they showed high levels of antibody titres, and none showed any clinical signs of disease (Hunter et al., 1998).

1.4.2.8 Elephant Endotheliotropic Herpesvirus (EEHV)

EEHV is a double stranded DNA herpesvirus found in African and Asian elephants both in the wild and in captivity (Fickel et al., 2001, Reid et al., 2006). The first noted case of a herpes like virus of elephants was found in the lungs of wild African elephants in Southern Africa (McCully et al., 1971).

In Asian elephants, EEHV is thought to be associated with haemorrhagic disease, and can result in sudden death. It is thought to be species specific; currently only elephants are thought to be affected this includes both African and Asian elephants. Clinical signs in Asian elephants include anorexia, lethargy, cyanosis of the tongue and sudden death (Ossent et al., 1990, Richman et al., 1999, Richman et al., 2000a, Ehlers et al., 2001, Garner et al., 2009, Sripiboon et al., 2013, Atkins et al., 2013).

In African elephants, clinical cases are rarely reported. The naming convention for the elephant-specific herpesvirus is

currently under much debate; many publications refer to the virus as Elephant Endotheliotropic Herpesvirus (EEHV), whereas others refer to the virus as EIHV (Elephantid herpesvirus) (Ehlers et al., 2006). The current taxonomic naming convention mentioned in The International Code of Virus Classification and Nomenclature refers to the virus as Elephantid herpes virus; however, the accepted acronym is not mentioned (Törün et al., 2005). Throughout this study it will be referred to as EEHV.

This virus will be discussed in greater detail in section 2.6.3.

1.5 Herpesviruses

1.5.1 Taxonomy

Herpesviruses are large linear double stranded, enveloped viruses; they are members of the *Herpesviridae* family. There have been around 200 herpesviruses described, but the actual number in nature is likely to be higher as many may be currently undetected due their life cycle. They are found in a wide range of species, including humans. Most species are host to multiple herpesviruses (Pellett and Roizman, 2007).

The *Herpesvirales* order contains three families. Alloherpesviridae, which contains herpesviruses known to infect fish and amphibians.

Herpesviridae, which contains three sub families (*Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*) of viruses known to infect mammals, reptiles and birds (Figure 4). There are 4 genera within each subfamily; *Alphaherpesvirinae* contains the following genera: Iitovirus, Mardivirus, Simplexvirus and Varicellovirus, Betaherpesvirinae: Cytomegalovirus, Muromegalovirus, Proboscivirus and Roseolovirus, and Gammaherpesvirinae,

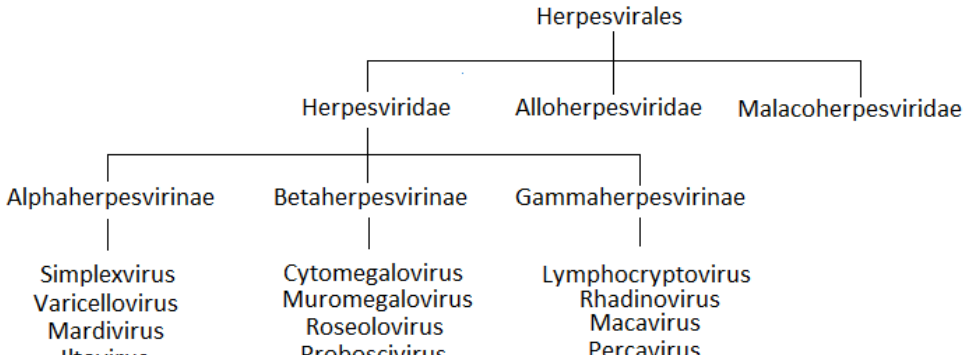

Figure 44: The order Herpesvirales including examples of viruses within each sub family. The above diagram is adapted from (Davison et al., 2009, Maclachlan and Dubovi, 2011)

Lymphocryptovirus, Macavirus, Percavirus and Rhadinovirus (Figure 4). Malacoherpesviridae contains herpesviruses known to infect bivalves. There are also a number of unassigned herpesviruses that have not been assigned to a particular family (Davison 2009).

For a virus to be included in the *Herpesviridae* family the primary criterion is virus morphology: it must have a spherical shape, and consist of four components which include the core,

the capsid, the envelope and the tegument. The core must contain a single copy of double-stranded, linear DNA which is packed into the capsid at a high density, and the capsid must also be icosahedral in shape (Mundy, 2014). Virus morphology is used to determine families, whereas serology (using neutralizing antibodies which are targeted against envelope glycoproteins) and genomics (to determine nucleic acid similarity) are used to determine how closely related the viruses are to one another (Mundy, 2014).

Viruses in the *Alphaherpesvirinae* genus have a short replication cycle and have a wider host range than the Beta and Gammaherpesviruses (Pellett and Roizman, 2007), *Alphaherpesvirinae* currently includes 36 species in 5 genera.

All Betaherpesviruses are highly species-specific in their infectivity. They have a long slow replication cycle in comparison to Alphaherpesviruses, and remain cell associated (contained within cells) (Mocarski Jr et al., 2007). The latent stage of the *Betaherpesvirinae* subfamily occurs within leukocytes (Pellett and Roizman, 2007). Betaherpesviruses are found in a number of animals including humans, there are currently 15 herpesviruses assigned to 4 genera in this genus including EEHV1.

Gammaherpesviruses have a narrow host range, and replication speed differs depending on the viral strain. Latency occurs within lymphocytes, and replication occurs within the epithelial cells of the host. *Gammaherpesvirinae* currently contains 29 species contained within 4 genera.

A fourth sub-family Deltaherpesvirinae has recently been suggested. Proposed viruses to be included within this sub - family are EEHV1A, EEHV1B and EEHV2, as it is thought that they evolved as a branch between Beta and Gammaherpesvirinae. EEHV is currently included in the Betaherpesvirinae sub-family along with a number of other betaherpesviruses of mammals. EEHV is included in the genus Proboscivirus, it is the only virus in this genus (Figure 5).

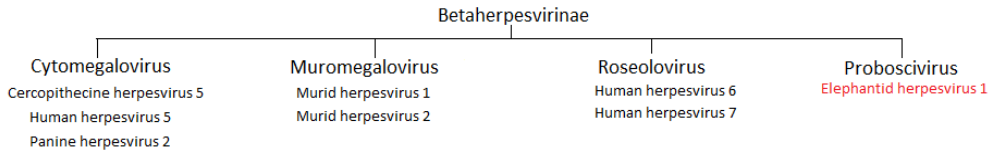

Figure 45: Sub family Betaherpesvirinae showing the position of Elephantid herpesvirus 1 within the four genera. The herpesvirus found in elephants is highlighted in red. Adapted from (Davison et al., 2009)

1.5.2 Structure

Herpesviruses can range in size from 120-260nm (Pellett and Roizman, 2007). The virion has several components including

the core, the capsid, the tegument and the envelope (Figure 6).

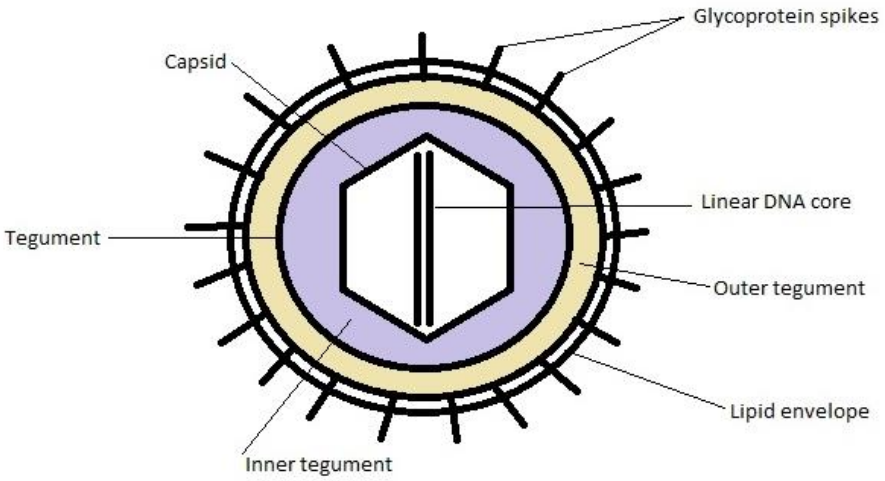

Figure 46: Herpesvirus structure diagram showing major components. The complex structure of a herpesvirus includes a large number of glycoprotein spikes attached to the lipid envelope, an outer tegument located between the tegument and the lipid envelope, the tegumet located between the inner and outer tegument, an inner tegument located between the tegument and the capsid, an icosahedral capsid and a DNA core containing linear DNA. Diagram adapted from Amen 2011.

The core consists of DNA wrapped around a torus, a cylindrical structure, 70nm in external diameter and 50nm in height (Furlong et al., 1972). The viral capsid consists of 162 capsomers (12 pentons and 150 hexons) and is icosahedral in shape; the role of the capsid is to encase and protect the DNA (Pellett and Roizman, 2007, Brown and Newcomb, 2011). The tegument is variable in size and shape and forms a layer around the capsid (Rixon, 1993). Mettenleiter et al. (2006) suggested that the tegument is split into two subcellular compartments, the inner and the outer tegument, with each

compartment containing a different array of proteins. The lipid envelope encases the complete virion, comprising of host cell lipids with a number of glycoprotein spikes protruding from the envelope surface (up to 14nm in length). A well-studied example, Human Herpes simplex virus, has three distinct types of spikes containing the glycoproteins: glycoprotein B (gB), glycoprotein C (gC) and glycoprotein D (gD). Their distribution across the envelope differs; gB spikes occur in clusters and are the most numerous, gC spikes occur randomly, and gD spikes appear to be irregularly distributed (Stannard et al., 1987).

1.5.3 Genome organisation

The core of a herpesvirus consists of viral DNA, which is linear and double stranded; the size of the DNA varies between 120-250 kilo-base pairs (kbp), depending on the species of virus (Pellett and Roizman, 2007). There are 40 conserved genes found in all alpha, beta and gamma herpesviruses, these are known as the core genes which encode for proteins involved in the metabolism of nucleic acid, structure of the virion and DNA replication, they are arranged in seven gene blocks (Table 2).

Table 2: Conserved gene blocks across all herpesviruses, HHV6 names and numbering are used as examples, source: Firyal and Naureen (2007).

Block	Genes	Properties
I	U27-U37	Tegument encoding proteins, DNA packing, capsid assembly and DNA polymerase gene
II	U38-U41	DNA polymerase gene, glycoprotein B gene and capsid assembly
III	U42-U46	DNA binding protein, tegument proteins, glycoprotein N gene and DNA helicase complex
IV	U48-U53	Glycoprotein gH, fusion protein, DNA packaging, G protein receptor gene, and capsid assembly protein gene
V	U56-U57	Major capsid protein
VI	U64-U77	Several virion proteins, helicase primase complex, Glycoprotein M and alkaline exonuclease
VII	U81-U82	Uracil-DNA glycosylase and Glycoprotein L

There are a number of genes that are unique to each subfamily, including a tegument associated gene and a transcriptional regulatory protein which are unique to the Alphaherpesviruses. Betaherpesviruses have a unique gene block (U2-U19) which is unlike any other within the Herpesvirus family; the gene block encodes a group of 14 proteins (Table 3). Gammaherpesviruses possess a unique gene block which encodes proteins essential for maintaining latency, whilst the genes for latency are spread throughout the genome for Alpha and Beta herpesviruses. There are also

a number of unique genes belonging to each viral species, and additional ones for the various strains within each species (Pellett and Roizman, 2007). These unique genes can be used to help identify the viral species and strains.

Table 3: Betaherpesvirus specific genes and their properties, sources: Gompels et al. (1995) and Dominguez et al. (1999).

Gene	Properties
U2	Tegument protein
U3	Tegument protein
U4	HCMV Maribavir resistance
U7	HCMV U22 gene family
U10	dUTPase family
U11	Antigenic tegument protein
U12	Chemokine G protein-coupled receptor
U13	Represses US3 transcription
U14	Antigenic tegument protein
U15	HCMV UL25 gene family
U17	Tegument protein
U18	Membrane glycoprotein
U19	Glycoprotein

The MHV-68 gene is found in Gammaherpesviruses such as EBV, it is a unique gene that encodes M2 latency associated protein. It may be used as a potential target to disrupt /prevent the establishment of latency (Husain et al., 1999).

1.5.4 Replication

1.5.4.1 Early and late phases

Replication of HSV1 has been studied extensively, and is the standard model used for the study of herpes viruses, and has been chosen for this review for this reason. However, it is likely there are variations in the cycle depending on the individual herpesvirus. The replication process will be explained for Herpes Simplex Virus 1 as it is a highly studied virus. Attachment to the host cell occurs when the herpesvirus glycoprotein spikes bind to the host cell receptors; the viral envelope then fuses with the cell plasma membrane (Pellett and Roizman, 2007). The envelope opens, and the capsid containing viral DNA is released and transported to the nucleus, via the nuclear pores. When viral DNA enters the nucleus, it causes host-cell macromolecule synthesis to stop. During the early phase, two different mRNA's are then transcribed to form α and β proteins, which are DNA binding proteins and enzymes. Firstly, α proteins are produced; this then initiates the transcription and translation of β proteins. Viral replication occurs using both the α and β proteins. In the late phase, γ proteins are produced after DNA synthesis has been initiated; these γ proteins are structural (Boehmer and Nimonkar, 2003, Pellett and Roizman, 2007, Cecil et al., 2008).

1.5.4.2 Viral DNA replication

DNA replication occurs within the nucleus. HSV has seven core genes essential for replication (Table 4): this includes UL9, a gene which is only found in alpha herpesviruses.

Table 4: Core HSV genes which are essential for DNA replication.

Gene	Abbreviation	Function	Present in
UL29	ICP8	Single stranded DNA-binding protein	All known herpesviruses
UL42		Processivity subunit of DNA polymerase	All known herpesviruses
UL5		Subunit of helicase/primase; contains helicase motifs	All known herpesviruses
UL8		Subunit of helicase/primase; interacts with other proteins	All known herpesviruses
UL52		Subunit of helicase/primase; contains primase motifs	All known herpesviruses
UL30		DNA polymerase	All known herpesviruses
UL9	OBP	Origin-binding protein, helicase	Alpha herpesviruses

Replication is initiated at one of the three viral origins of replication (Ori 1, Ori2 and Ori 3) which are specific areas within the genome. UL9 an origin binding protein which contains DNA helicase motifs and an ATP binding site then unwinds and binds to the DNA (Taylor et al., 2002). ICP8 a

major single stranded DNA binding protein is attached to the unwound DNA. HSV1 Helicase/primase complex a complex heterodimer, and a DNA polymerase holoenzyme (a heterodimer) is then recruited to the replication forks, and bind to DNA (Taylor et al., 2002). Synthesis of DNA proceeds via a theta replication mechanism. During the replication to a circular DNA molecule, a theta structure is formed. Two replication forks are formed; these are created from helicases breaking bonds holding the double stranded DNA together, this results in a section of double stranded DNA with two single stranded DNA prongs at the end. The prongs serve as the leading strand template and the lagging strand template (Waga and Stillman, 1998, Johnson and O'Donnell, 2005). Replication then switches to a rolling circle replication mechanism, which is a unidirectional process of nucleic acid production, where synthesis occurs rapidly. It is thought that replication is swapped to the rolling circle mechanism to enable to majority of progeny genomes to be synthesised (Jacob et al., 1979) (Figure 7).

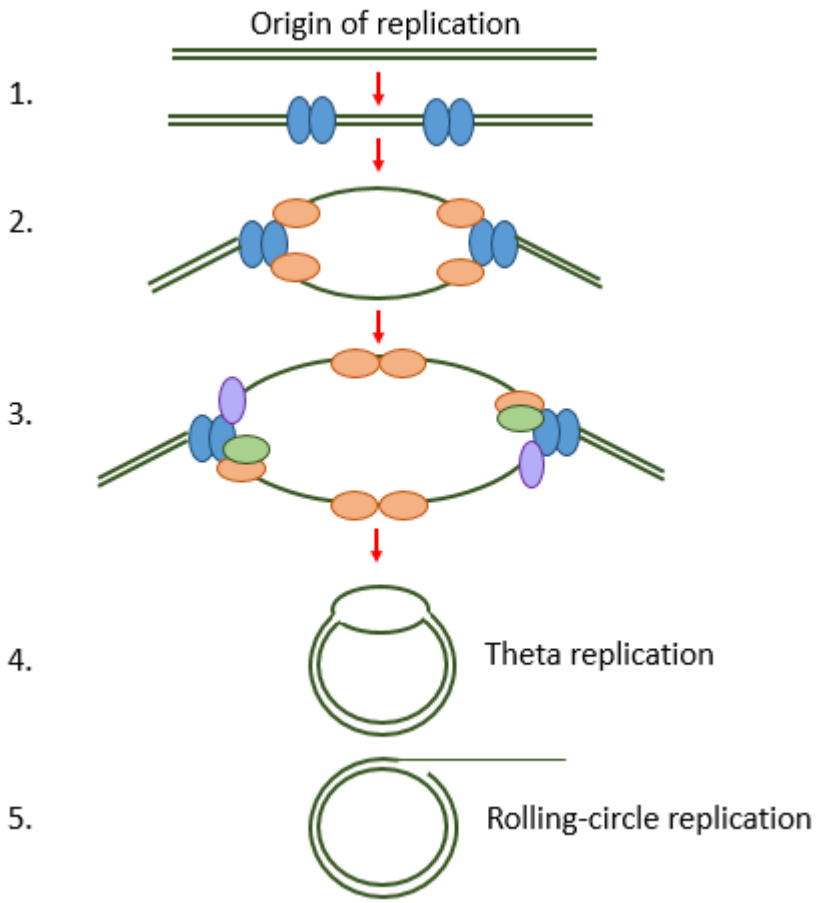

Figure 47: HSV DNA replication adapted from Taylor et al. (2002). There are five main stages of HSV DNA replication: 1, UL9 (blue circle) binds to the DNA and begins to unwind it, this occurs at a specific site at an origin of replication, 2, ICP8 (orange circle), a single stranded binding protein is then recruited to the unwound DNA, 3, IPC8 and UL9 then recruit the helicase/primase complex (purple circle) and DNA polymerase holoenzyme (green circle) to the replication forks, 4, DNA synthesis begins via a theta replication mechanism, 5, replication then switches to a rolling circle mechanism.

1.5.4.3 Viral protein production

For viruses to be able to reproduce, they must produce proteins, and therefore they must transcribe and translate mRNAs from their genomes. The mechanism used varies depending on the viral family. Important proteins made by viruses include viral immunomodulatory proteins which are used to evade the immune response (Husain et al., 1999).

1.5.4.3.1 Viral immunomodulatory proteins

Virokines (ligand like) and viroreceptors (receptor-like) are immunomodulatory proteins which are encoded by some large DNA viruses, including herpesviruses (Husain et al., 1999, Lucas and McFadden, 2004). They are produced by the host cell, and are used by the virus to evade the host's acquired and innate immune response. Virokines are able to occupy the binding site, this process uses competitive inhibition of host signalling molecules, the result of this means signalling molecules are then unable to access their target site. The virokine is then sequestered by the cytokine binding protein, obstructing the binding site. The host's immune response can be reduced as interaction with receptors has been interrupted (Reddacliff et al., 1997). Another way in which viruses are able to evade the host's immune system is the modulation of chemokine activity through the expression of type 1 transmembrane proteins called viral chemokine binding proteins. The viral chemokine binding proteins bind to chemokines and are then able to modulate their activity (González-Motos et al., 2016).

1.5.4.4 Virion assembly and egress

In the generic herpesvirus replication cycle, assembly of the new virion occurs within the nucleus of the host cell. The newly replicated viral DNA is packaged into a new capsid and

matures; infectivity is acquired via budding through the nuclear membrane (Pellett and Roizman, 2007). The new virion is transported through the Golgi apparatus, where glycosylation occurs: glycans are attached to the lipid envelope, creating glycoprotein spikes (Cecil et al., 2008). Finally, egress occurs when the virion is transported to the cell membrane; the progeny exit the cell via the exocytosis pathway. When viral exit or egress occurs, progeny are released and subsequently cause host cell death (Hunt, 2013) (Figure 8).

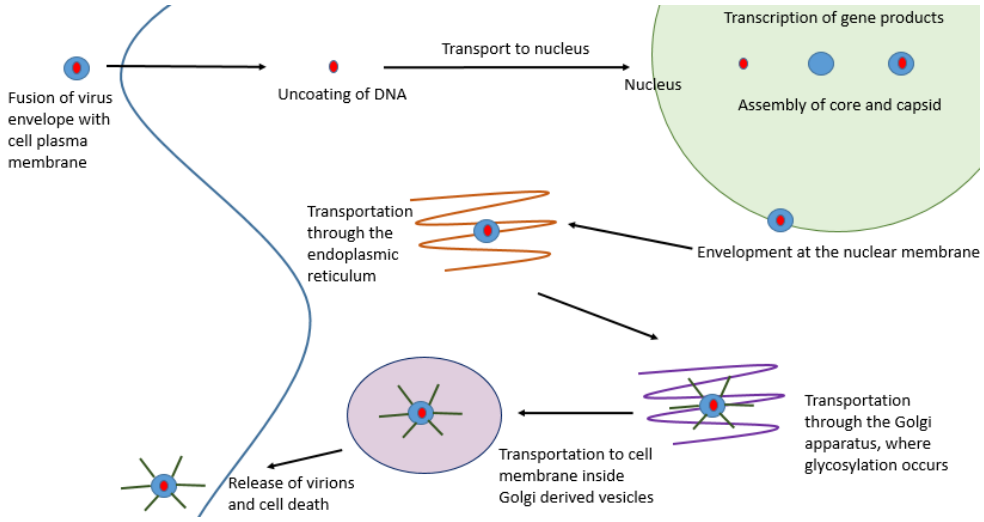

Figure 48: Herpes virus replication cycle adapted from Cecil et al. (2008). The herpesvirus enters the host cell; the capsid disintergrates releasing viral DNA, which is then transported to the nucleus where transcription occurs. The viral DNA is encapsulated within a capsid, and the virion is transported through the endoplasmic reticulum and golgi apparatus. Within in the Golgi apparatus, the virion undergoes glycosylation, which is a process where glycans are attached. The virion is then transported to the cell membrane inside Golgi derived vesicles. The new virion progeny is then released from the cell, and the host cell subsequently dies.

1.5.5 Latency and reactivation

All herpesviruses are capable of maintaining latency within the host's cells; where this occurs depends upon the species of herpesvirus. Alphaherpesviruses, such as HSV and VZV in humans, establish and maintain latency within neurons, which are non-dividing cells. This is achieved as the virus migrates into the neurons in the peripheral tissue. The virus then invades the nucleus and becomes circularized; the genome is then maintained in this form (Gupta et al., 2008). HSV remains completely inactive during latency, whereas VZV does not, some proteins are still able to be detected, these include intermediate-early and early proteins. During an active infection these proteins are intranuclear, whereas during a period of latency these proteins accumulate in the cytoplasm of the infected neurons (Van Eys et al., 1989). It is thought that HSV1 avoids cell death by restricting viral gene expression; the only gene transcribed during HSV latency is latency associated transcript (LAT). LAT is RNA which accumulates within the nuclei of infected cells. LAT is able to interfere with the normal function of the infected cell, it also regulates viral gene expression (Block and Hill, 1997).

Infection with a herpesvirus can result in a lifelong infection with long periods of viral latency; viral reactivation may occur during stress or low immunity and result in clinical disease.

When reactivation of the herpesvirus occurs through events such as stress, lytic replication is resumed, and the infected cell and its membrane is destroyed. The virus reactivates within the neuron, and is transported to the skin or mucosal surface. Lesions caused by herpesvirus infection are not limited to the initial site of infection, as the virus is able to travel along the axon and exit the nerve at any branch of the axon (Gupta et al., 2008).

1.5.6 Herpes induced disease

Herpesviruses cause a wide range of symptoms and diseases in both humans and animals (Table 5). Humans are currently the only host species that can be infected with herpesviruses from all subfamilies, there are eight species known to infect humans. Human herpesviruses include HSV1 and HSV2, both belonging to the subfamily *Alphaherpesvirinae*, and known to cause ulceration on the genitals or oral mucosa, though an attack of genital herpes is more commonly associated with HSV2 (Fleming et al., 1997, Lafferty et al., 2000, Pellett and Roizman, 2007). Varicella Zoster virus is also an alphaherpesvirus and is the causative agent of chicken pox

and shingles, also common among humans (Pellett and Roizman, 2007). Symptoms of chickenpox are commonly a pruritic vesicular rash which can be located in widespread areas on the body. Reactivation of VZV leads to shingles, the rash is localised and painful (Arvin, 1996).

Epstein-Barr virus (EBV) is a gammaherpesvirus which is thought to be associated with carcinomas and lymphomas such as Burkitt's lymphoma (zur Hausen et al., 1970, Lindahl et al., 1974, Pellett and Roizman, 2007). Patients infected with EBV may show symptoms such as fever, cough, rashes, and lymphadenopathy (Ellis, 2014). EBV has also been associated with infectious mononucleosis (glandular fever) and tonsillitis, the symptoms of which include pharyngitis, malaise, splenomegaly, hepatomegaly and jaundice (Shigeta et al., 1983, Kleymann et al., 2002, Bailey, 1994, Rothman, 2002).

Human cytomegalovirus (HCMV) is a betaherpesvirus found in up to 90% of adults, most adults are asymptomatic and people rarely experience fever and/or mild hepatitis (Staras et al., 2006). Reactivation of the virus and a recurrent infection are rare unless the person is immunocompromised, where the following symptoms may be observed: retinitis, nephritis and cystitis (Ljungman et al., 2002). Human herpesviruses 6, 7

and 8 are less studied. HHV6 and 7 are members of the betaherpesvirus subfamily, HHV6A has been associated with multiple sclerosis, HHV6B and HHV7 have been associated with exanthema subitum also known as sixth disease, clinical symptoms include rashes on the trunk spreading to other parts of the body and fever (Yamanishi et al., 1988, Tanaka et al., 1994, Challoner et al., 1995, Amo et al., 2003). The eighth human herpesvirus is HHV-8, has been associated with Karposi's sarcoma (Gillison and Ambinder, 1997).

There are a large number of important herpesviruses in animals, of which only some will be discussed (Table 5). A number of domestic species such as cattle, horses and dogs can be affected. Cattle can be infected with Bovine herpesvirus 1 (BVH1) known to cause rhinotracheitis, vaginitis and abortions (National Research Council Committee on Methods of Producing Monoclonal Antibodies, 1999, Quignot et al., 2014). Equid herpesvirus 1 (EHV1) affects both domestic and wild equids, and is known to cause abortions and neurological problems (Edington et al., 1991, Mumford et al., 1994, Borchers et al., 2006). Canine herpesvirus 1 affects both domestic dogs and wild canines, and was first associated with fatal haemorrhagic disease in 1965, it has since also been associated with fertility disorders such as embryonic

reabsorption and neonatal mortality (Carmichael et al., 1965, Ronsse et al., 2002, Ronsse et al., 2004, Ronsse et al., 2005). Alcelaphine Herpesvirus 1 (AIHV1) is associated with bovine malignant catarrhal fever, the clinical signs of which include fever, ocular and nasal discharge, lesions in the mouth and vasculitis, death may occur within several days (Liggitt and DeMartini, 1980, Schultheiss et al., 1998, Schultheiss et al., 2000). Neurological signs may also be present in some animals including ataxia, corneal opacity may occur which can lead to blindness (Albini et al., 2003, Mitchell and Scholes, 2009). Whilst the natural host for AIHV1 is cattle, wildebeest act as an asymptomatic reservoir host and AIHV1 is thought to derive from the Wildebeest (Wambua et al., 2016). Another virus which is associated with malignant catarrhal fever is Ovine Herpesvirus 2 (OVHV2) which is thought to cause sheep associated malignant catarrhal fever. OVHV2 is contracted via contact with sheep; the primary mode of transmission is thought to be via nasal secretions (Li et al., 2004, Berezowski et al., 2005).

A number of different animal species such as pigs, cattle and bison have been known to be infected with the virus, which can cause large outbreaks (Reid and Pow, 1998, Schultheiss et al., 2000, Simon et al., 2003).

Table 5: Important herpesvirus of humans and animals including disease association, this list is not exhaustive. Information sourced from (Braun et al., 1997, Pellett and Roizman, 2007, Ernest et al., 2000).

Herpesvirus	Primary host	Disease association
Human Herpesvirus 1 (HSV-1)	Humans	Oral/genital herpes
Human Herpesvirus 2 (HSV-2)	Humans	Genital herpes
Human Herpesvirus 3 (VZV)	Humans	Chicken pox and shingles
Human Herpesvirus 4 (EBV)	Humans	Associated with Burkitt's lymphoma and nasopharyngeal carcinoma
Human Herpesvirus 5 (HCMV)	Humans	Major cause of deafness and retardation
Human Herpesvirus 6A (HHV-6)	Humans	Possible association of HHV-6A and multiple sclerosis. HHV-6B is associated with exanthema subitum
Human Herpesvirus 7 (HHV-7)	Humans	Associated with exanthema subitum
Human Herpesvirus 8 (HHV-8)	Humans	Associated with Karposi's sarcoma
Bovine Herpesvirus 1 (BHV-1)	Cattle	Rhinotracheitis, vaginitis and abortion
Equine Herpesvirus 1 (EHV-1)	Equids	Respiratory disease and neonatal mortality
Alcelaphine Herpesvirus 1 (AIHV-1)	Wildebeest and Hartebeest	Bovine malignant catarrhal fever
Canine herpesvirus 1 (CHV-1)	Canines	Fatal haemorrhagic disease

1.5.7 Cross species transmission of herpesviruses

Many herpesviruses are host specific; however, a few are epizootic and zoonotic. Equine herpesvirus 1 and 9 are examples of such; both are alphaherpesviruses and are members of the *Varicellovirus genus.* Equine herpesvirus 1 (EHV1) is the causative agent of respiratory illnesses in

horses, and is associated with abortion or neurological disease. EHV1 has been isolated in a number of other species, including other equids such as various subspecies of Zebra (*Equus quagga, Equus quagga boehmi*, *Equus quagga burchellii* and *Equus grevyi*) and Persian onager all of which were isolated from captive animals (Borchers et al., 2005, Ghanem et al., 2008, Ibrahim et al., 2007, Guo et al., 2014). Many cases of fatal encephalitis linked to the presence of EHV1 have occurred in non-equids, including guinea pigs (captive), Thompson's gazelle (captive), Indian rhinoceros (captive), black bears (captive), camels (captive) and llamas (experimental captive infection) (House et al., 1991, Bildfell et al., 1996, Wohlsein et al., 2011, Abdelgawad et al., 2013). There are also cases of zoonotic transmission of Herpes simian B virus, the natural host of which is macaque monkeys. Herpes B virus is also an alphaherpesvirus, in the genus *Simplexvirus*. Since the discovery of Herpes B virus in the 1930's there have been around 40 documented human infections (Fu and Fu-Liu, 2002). Clinical signs in macaques include conjunctivitis, genital ulcers and gingivostomatitis causing mild disease; in humans, signs can include weakness, numbness, dizziness, conjunctivitis, flu-like symptoms, headache, and burning dysesthesia, and in some cases fatal encephalomyelitis can occur (Davenport et al., 1994).

A number of cases have been reported in those that have close contact with macaques, such as veterinary and laboratory staff. Transmission in these cases has occurred by needlestick injury, bites and scratches (Sabin and Wright, 1934, Breen et al., 1958, Davenport et al., 1994, Artenstein et al., 1991).

1.5.8 Herpesvirus infections during pregnancy

In humans, acquiring a herpesvirus infection during pregnancy can have major health consequences; it has been associated with spontaneous abortion, congenital herpes, neonatal herpes and prematurity. It has been reported that 2% of women acquire HSV infections during pregnancy (Brown et al., 1997). The time of acquisition may also be significant: acquisition within 6 weeks of labour is often associated with neonatal herpes (Brown et al., 1997). There is some evidence of low birth weight and prematurity if HSV1 or 2 is acquired during the third trimester. Caesarean section is recommended for women with genital herpes to prevent the new-born coming into contact with lesions during birth (Tsara et al., 2009).

1.5.9 Diagnostic methods

Herpesvirus infections can be diagnosed by a variety of methods, including: direct detection of viral proteins or DNA through ELISA and PCR, detection of the host's immune

response to the virus, and viral culture. Viral culture is used in the detection of genital HSV, and is described as the gold standard method, whereas PCR is used for HSV of the central nervous system (Frantz et al., 2003). However, it can be difficult to amplify DNA in some samples using PCR due to the presence of inhibitors, such as faecal and blood samples (Monteiro et al., 1997, Al-Soud et al., 2000).

For culture, a wide variety of samples can be used such as swabs of skin lesions, vesicular fluid from a lesion or a mucosal sample of an area with a lesion. However, culturing the virus is less sensitive than other methods, such as PCR, and results can take up to 7 days. Cultures must also be conducted in specialised laboratories, sample transport conditions and storage can affect the sensitivity (Yeager et al., 1979). Molecular methods can be used to detect viral DNA of HSV, including PCR. The advantage of using PCR is its high sensitivity, but it is not quantitative and cannot measure the amount of virus present unless qPCR is used (Slomka et al., 1998, Ryncarz et al., 1999, Engelmann et al., 2008).

Several serological methods for the detection of antiviral antibodies have been used in detecting HSV, including Western blotting for HSV1 (University of Washington, 2017). This is the gold standard test proposed by some institutions, but it is not commercially available. ELISA may also be used

on serum samples, but in some circumstances there may be issues with both sensitivity and specificity (Baker et al., 1989, Turner et al., 2002, Elshal and McCoy, 2006).

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1.5.10 Treatment

Several drugs are available for the treatment of Herpesvirus infections; these include nucleoside analogues such as famciclovir, aciclovir, ganciclovir, vidarabine, valganciclovir and valaciclovir, cidofovir, trifluridine, and idoxuridine. Other drugs include foscarnet which is derived from phosphoric acid and fomivirsen a synthetic oligonucleotide. Administration of antivirals is achieved via a variety of routes including oral, intravenous, topical and as eye drops, this varies depending on the drug (De Clercq, 2004) (Table 6).

Table 6: Drugs used to treat Herpesvirus infections. Information sourced from (De Clercq, 2004) **.**

	Orally	**Intravenous**	**Topical**	**Eye drops**
Penciclovir			Yes	
Famciclovir	Yes			
Ganciclovir	Yes	Yes		
Aciclovir	Yes	Yes	Yes	Yes
Valalciclovir	Yes			
Valganciclovir	Yes			
Cidofovir		Yes		
Vidarabine		Yes		Yes
Trifluridine				Yes
Idoxuridine				Yes
Foscarnet		Yes		
Formivirsen		Yes		

1.5.10.1 Nucleoside analogues

Nucleoside analogues include vidarabine, aciclovir, penciclovir, famciclovir, ganciclovir, valaciclovir, valganciclovir, cidofovir, trifluridine, and idoxuridine. They are initially phosphorylated by a nucleoside kinase such as herpesvirus thymidine kinase, which leads to the production of a monophosphate metabolite. A nucleoside monophosphate kinase then performs a second phosphorylation; a final third phosphorylation step is then completed by a nucleoside diphosphate kinase. The triphosphates can either inhibit essential enzymes which then inhibit nucleic acid synthesis, or they can be incorporated into the nucleic acids, where there is then competitive inhibition with their normal counterparts, which then prevents the virus from replicating (Jordheim et al., 2013) (Figure 9).

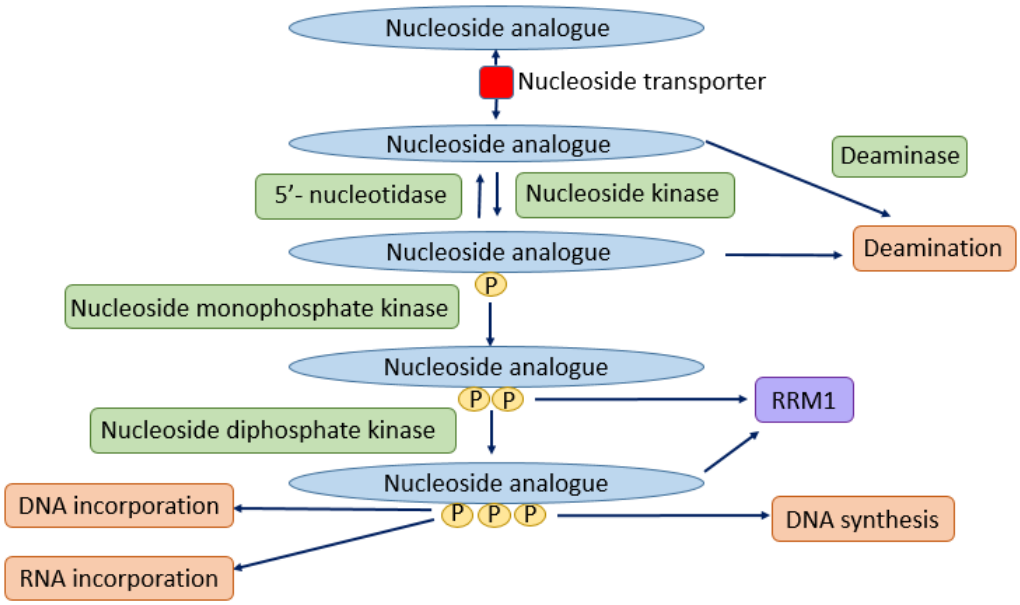


Figure 9: The mechanism of action of nucleoside analogues. Image adapted from Jordheim et al. (2013). The process of the cellular uptake of nucleoside analogues involves concentrative nucleoside transporters (CNT's) and equilibrative nucleoside transporters (ENT's). When the nucleoside analogue is within the cell it undergoes phosphorylation step where a phosphate is added this is performed by a nucleoside kinase, this leads to a monophosphate metabolite. A second phosphate is added, this is performed by a nucleoside monophosphate kinase, and a third phosphate is added this is performed by a nucleoside diphosphate kinase. An enzyme called Ribonucleotide reductase 1 (RRM1) is involved in nucleotide metabolism, dephosphorylated and triphosphorylated analogues can inhibit RRM1. The amount of active metabolites can be reduced by catabolic enzymes such as deaminases and 5'-nucleotidases.

1.5.10.2 DNA polymerase inhibitors

The antiviral drug Foscarnet is a DNA polymerase inhibitor which structurally mimics the anion pyrophosphate (Meyer et al., 2007). This drug works by inhibiting the pyrophosphate binding site on the viral DNA polymerases. The concentrations do not affect human polymerases, meaning the drug is less toxic than nucleoside analogues. There are advantages when using this drug: as it is not activated by viral protein kinases such as thymidine kinase, making it useful in the treatment of immunocompromised patients and in aciclovir or ganciclovir

resistant herpesvirus infections (Trial, 1989, Safrin et al., 1991, Jacobson et al., 1991, Morfeldt and Torssander, 1994, Mylonakis et al., 2002). However, mutants may also be resistant to Foscarnet (Weinberg et al., 2003). The major disadvantage of Foscarnet in the treatment of HCMV infections in transplant patients, is its renal toxicity, which can lead to renal impairment (Lischka and Zimmermann, 2008).

1.5.10.3 Synthetic oligonucleotide

Fomivirsen is a synthetic oligonucleotide, and is also resistant to degradation by nucleases. It blocks the translation of viral mRNA by binding to the complementary sequence of mRNA (Malik and Roy, 2008). It is currently used in the treatment of HCMV, especially in immunocompromised patients as this drug does not target viral DNA polymerases.

1.5.10.4 Vaccines

For most herpesviruses, vaccines are not available; however, in humans, a successful vaccine has been created for VZV. The vaccine has been considered a success in both Germany and the USA. In Germany, between 2005 and 2009, there was a reduction in the number of chicken pox cases by 55% in all age groups (Siedler and Arndt, 2010). Between 1995 and 2005 at two sites in the USA, varicella incidence decreased by 90%; reductions were observed in all age groups (Guris et al.,

2008). The herpes zoster vaccine ZOSTAVAX (Merck) is available in a number of countries including the UK.

There have been a number of attempts to develop vaccines for other herpesviruses, specifically HSV1 and 2. One of these was Herpevac, a proposed vaccine against HSV2; in 2002 it was reported to be effective in women who were seronegative for HSV1 (Jacob et al., 1979). In 2010, a phase 3 trial for Herpevac conducted a study of 8323 women aged between 18-30 and free of both HSV1 and 2. Each participant was vaccinated at the beginning of the study, and again 6 months later. After the initial injection participants were followed for 20 months, and were evaluated for the presence of both HSV-1 and 2. The study concluded that the vaccine was not commercially viable, as it was only effective in certain women and designed for a very narrow market (Galimand et al., 2006).

1.5.11 Control strategies

A number of herpesviruses are classed as notifiable diseases, and the correct authority must be notified when a case occurs. Herpesviruses of animals are also included on the notifiable list in England/Wales and Scotland, with Koi herpesviruses needing to be reported in both England, Wales and Scotland, and Ostried herpesvirus in Scotland.

Vaccination against VZV at an early age can prevent the number of cases of chicken pox. Other advice is given to those with active herpesvirus infections to prevent the spread of the virus to others. This includes not taking children infected with VZV to school whilst they have weeping pustules, and for people infected with HSV, not sharing utensils or kissing others whilst they have a cold sore. A vaccine is also available for the prevention of equine herpesvirus 1 (EHV1) and 4 (EHV4), it is suggested that vaccination should begin when foals are between 3-4 months old, second and third doses are also required at one month intervals, however, this may be different if the mare was not vaccinated (McCue, No date). Another vaccine is usually recommend for EHV1, an inactivated vaccine is given at 3, 5, 7, 9 months through pregnancy, this is to prevent EHV1 related abortion (McCue, No date).

1.6 Herpesviruses of elephants

Based on current literature three herpesviruses have been found in elephants, including bovine herpes, Elephant Gammaherpesvirus (EGHV) and EEHV (Metzler et al., 1990, Ossent et al., 1990, Latimer et al., 2011).

1.6.1 Bovine herpes

Serological techniques have been used for the detection of Herpesvirus antibodies in elephants. Metzler et al. (1990) studied 17 captive Asian elephants for the presence of bovine herpesvirus antibodies using a number of serological tests. Sera used included pericardial fluid, blood and lyophilized serum samples. Samples were tested for antibodies to BHV1, BHV2 and BHV4. Serological tests included a seroneutralisation test, radio-immunoprecipitation and sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE), and antibodies for 3 bovine herpesviruses were found in all elephants tested. The titres for bovine herpesvirus were at low levels, the study also suggests that this level was potentially low due to the serology being conducted with heterologous viruses, which potentially meant only cross-reacting antibodies were detected. However, the evidence from this study may not be robust and is limited as the study highlights that there may be a limitation as BHV2 may have been neutralized by the generation of antibodies from a HSV infection (passed from humans) however, this is unlikely, repeat or duplicate samples are also not described which limits the validity of the results. The study included samples from 21 captive Asian elephants, sera would need to be obtained from

more captive and free ranging Asian and African elephants to provide conclusive evidence.

1.6.2 Elephant gamma herpesviruses

There are six Elephant Gammaherpesviruses: Elephant Gammaherpesvirus 1 (EGHV1), EGHV2, EGHV3, EGHV4, EGHV5 and EGHV6. There are two strains of EGHV3: EGHV3A and EGHV3B. EGHV3B is found in African elephants and EGHV3A is found in Asian elephants. These have been detected (in conjunctival and genital secretions of asymptomatic animals) using PCR to amplify the DNA polymerase gene (Wellehan et al., 2008, Latimer et al., 2011). EGHVs have not been associated with clinical disease, and their prevalence among different elephant populations is unknown. However, Latimer et al. (2011) reported cases in which there was a co-infection of EEHV and EGHV, and Masters et al. (2011) documented a case in which EGHV5 was detected by PCR in a biopsy sample from a mass in the nostril of a 26 year old female Asian zoo elephant.

1.6.3 Elephant Endotheliotropic Herpesvirus (EEHV)

1.6.3.1 EEHV phylogeny

EEHV is a virus of both African and Asian elephants. It was previously suggested that EEHV originated in the African elephant and spread when African and Asian elephants came

together or were co-housed in a zoo environment (Richman et al., 1999, Richman et al., 2000b). This is no longer the accepted theory, as EEHV has been found in Asian elephants with no previous contact with African elephants, and at high prevalence rates in Asian elephant range countries (Stanton et al., 2014).

1.6.3.2 Variants

There are thought to be several variants of EEHV. Two of the variants, EEHV1 and 2 have been identified based on sections of the U60 terminase gene and the U38 DNA polymerase gene. Further analysis has shown two chimeric variants of EEHV1, named EEHV1A and 1B. Although much of their sequence is very similar, differences can be seen in the glycoprotein B, O and H genes and the thymidine kinase gene (Ehlers et al., 2001, Fickel et al., 2001, Ehlers et al., 2006). EEHV3A and 3B are within a GC rich (67%) branch of the Probosciviruses, this is contrasted with the AT rich branch which includes EEHV1, 2, 5 and 6 (Latimer et al., 2011).
It is thought that EEHV1,3,4, and 5 are all present in Asian elephant populations and EEHV 2,3,6 and 7 are present in African elephant populations (Hayward, 2012). For reasons that are poorly understood, most fatal cases of EEHV have been linked to EEHV1; deaths relating to EEHV4 and 5 have

also been reported but are thought to be rarer (Richman et al., 2000a, Denk et al., 2012, Sripiboon et al., 2013).

1.6.3.3 EEHV in African elephants

Nodular lesions associated with herpesvirus infection were first detected in African elephants in 1971 (McCully 1971); more recent research has identified multiple EEHV's within the lung and splenic tissue of asymptomatic African elephants in South Africa, USA, Kenya and Spain. Samples from two wild elephants in South Africa were both positive for EEHV2. Of the elephants located in the USA, one was positive for EEHV6, one for EEHV3, one for EEHV2 and one for EEHV7. Of the four animals located in Kenya, each of the animals tested positive for EEHV, EEHV2, EEHV3, EEHV6 or EEHV7. Samples from 3 African elephants located in Spain each tested positive for a different EEHV: EEHV2, 3 or 6 (Centers for Disease Control, 2015).

1.6.3.4 EEHV in Asian elephants

1.6.3.4.1 Clinical disease

EEHV has a mortality rate of 85% in calves that develop clinical signs (Latimer et al., 2011). From 1992-2012, 24% of elephant calves in the USA were affected by EEHV (Hayward, 2012).

EEHV is associated with haemorrhagic disease in Asian elephants. The onset of clinical disease is extremely rapid. There are a number of clinical signs including: lethargy, oedema of the face, neck and trunk, oral ulceration, cyanosis of the tongue, reluctance to eat, depression, decreased stool, tachycardia and anorexia (Ossent et al., 1990, Richman et al., 1999, Richman et al., 2000a, Ehlers et al., 2001, Garner et al., 2009, Sripiboon et al., 2013, Atkins et al., 2013). Post mortem examinations have revealed a number of anomalies including: pericardial effusion, haemorrhages (in the small intestine, large intestine, heart, stomach, lungs, trunk, tongue, lymph nodes and kidneys), pulmonary oedema, congestion, ulceration and cyanosis of the tongue (Ossent et al., 1990, Richman et al., 1999, Reid et al., 2006, Garner et al., 2009, Sripiboon et al., 2013, Zachariah et al., 2013).

1.6.3.4.2 Reported cases to date

Up to January 2016 22 cases have been reported this includes 19 deaths and 3 survivors, 13.6% of the cases have occurred in Europe, 27.3% in USA and 59.1% in Asia, the greatest number of deaths (63.2%) occurred in those aged below 5. Ten of the cases used multiple diagnostic methods, including PCR, serology, pathology and histology, whereas 12 cases were diagnosed using PCR only.

A point of interest from the data gathered in Table 7 is that there are no deaths below the age of 1 year, it is uncertain if this related to the presence of maternal antibody during the first year, it is not currently known if primary exposure occurs when the juvenile has maternal antibodies, are they then able to develop their own immunity to the virus in later life.

Table 7: Reported cases of EEHV infection in Asian elephants.

Species	Status	Date of death	Method of diagnosis	EEHV variant	Age	Location	Reference
Asian	Survived	N/A	PCR, serology, pathology	N/A	17 months	USA	(Richman et al., 2000a)
Asian	Survived	N/A	PCR, serology, pathology	N/A	16 months	USA	Richman et al., 2000a
Asian	Survived	N/A	PCR	EEHV 5	42	USA	(Atkins et al., 2013)
Asian	Deceased	N/A	Histology and pathology	N/A	3	Switzerland	Ossent 1990
Asian	Deceased	Apr-95	PCR, serology, pathology	N/A	16 months	USA	Richman 2000a
Asian	Deceased	Aug-98	PCR	EEHV 1	11	Germany	Ehlers 2001
Asian	Deceased	Unknown	PCR, pathology, histology	EEHV 3	5	USA	Garner 2009
Asian	Deceased	Unknown	PCR, pathology, histology	EEHV 3	6.5	USA	Garner 2009
Asian	Deceased	Unknown	PCR, pathology, histology	EEHV 5	20 months	UK	Denk 2012
Asian	Deceased	Jan-05	PCR, pathology, histology	EEHV 1A	2	Thailand	Siripboon 2013
Asian	Deceased	Apr-11	PCR, pathology, histology	EEHV 4	3	Thailand	Siripboon 2013
Asian	Deceased	May-04	PCR	EEHV 1	3	Cambodia	Reid 2006
Asian	Deceased	2005	PCR	EEHV 1	12	India	Zachariah 2013
Asian	Deceased	2007	PCR	EEHV 1	5	India	Zachariah 2013
Asian	Deceased	2007	PCR	EEHV 1	2	India	Zachariah 2013
Asian	Deceased	2007	PCR	EEHV 1	2	India	Zachariah 2013
Asian	Deceased	2008	PCR	EEHV 1	6	India	Zachariah 2013
Asian	Deceased	2009	PCR	EEHV 1	8	India	Zachariah 2013
Asian	Deceased	2010	PCR	EEHV 1	8	India	Zachariah 2013
Asian	Deceased	2011	PCR	EEHV 1	18 months	India	Zachariah 2013
Asian	Deceased	2011	PCR	EEHV 1	7	India	Zachariah 2013
Asian	Deceased	Feb-11	PCR, pathology, histology	EEHV 1A	30 months	Laos	Bouchard 2014

1.6.3.4.3 Current recommendations and treatment

There are no set guidelines for treating EEHV; however, there are published protocols available. A protocol from the United Kingdom Elephant Health Programme was created in response to an EEHV related death in the UK, and was used globally (Cracknell, No date). Another protocol is available from the Houston Zoo. Both recommend treatment with anti-herpetics, antibiotics, analgesics, fluid therapy and oxygen therapy. However, The Elephant Health Programme recommends vasoconstrictors in the management of circulatory failure and the use of diuretics, whereas the Houston Zoo recommends plasma transfusion when the sick animal blood type is matched with a correct donor, and a blood transfusion when haematocrit levels fall below 14% (Table 8) (Houston Zoo, 2010).

Table 8. A comparison of two EEHV treatment protocols.

Recommendations:	**United Kingdom Elephant Health Programme EEHV protocol (Cracknell, No date)**	**Houston Zoo EEHV protocol (Houston Zoo, 2010)**
Diagnostic testing	PCR or ELIZA	Complete blood count, Serum biochemical analysis, and PCR
Antiherpetic drugs suggested	Famciclovir – orally or rectally	Famiciclovir (orally or rectally), or Ganiciclovir (orally)
Antibiotics	Recommended to prevent infection, a number of drugs are available for use in elephants: Amoxicilin, Ampicillin, Ceftiofur, Doxycycline, Enrofloxcin, Marbofloxacin, Procain pencillin and Benzathine penicillin	Recommended to prevent infection and to be instigated immediately (list of antibiotics in protocol)
Analgesia	Non-steroidal anti-inflammatories and opioids may also be used	Non-steroidal anti-inflammatories and opioids may also be used
Fluid therapy	Yes recommended	Rectal or intravenous fluid therapy recommended
Oxygen therapy	Yes recommended – flow rate of 5 litres	Administered where possible
Diuretics	Flurosemide	N/A
Pericardial tap	Yes recommended	N/A
Vasoconstrictors	Can be used where blood pressure can be measured or as a last resort	N/A
Daily herd management	Oral examination (ulcers, tongue lesions), general assessment and faecal ball temperature	Oral examination, general inspection of the animal, appetite assessment, temperature, and blood pressure assessment (twice for all elephants and daily for calves)
Daily monitoring of an EEHV case	N/A	Blood samples to be taken daily during the first week of treatment, daily urine samples and faecal samples
Plasma transfusion	N/A	If available
Blood transfusion	N/A	Should be considered if haematocrit falls below 14%

The EEHV Advisory Group is a group of EEHV specialists consisting of zoo staff, veterinary surgeons and scientists. Their mission is to "decrease elephant mortality and morbidity due to EEHV" (Hájková et al., 2006). Their goals are to recommend treatment and management protocols, coordinate research samples and fund-raising (Hájková et al., 2006). The group has produced a document stating the minimum standards of care for elephant calves in relation to EEHV preparedness. Recommendations are made for calf training to enable successful sample collection for EEHV detection and monitoring. By the age of one year, calves should be trained for voluntary blood collection, trunk wash collection, standing on scales, oral exam, ocular exam, faecal bolus temperature measurement, blood pressure and standing for monitoring of the pulse. Calves should also be trained by one year of age to be separated from the dam, restrained, be able to receive medications orally and rectally, accept fluids rectally and orally from a bottle and be able to receive medications via injection (EEHV Advisory Group, No date).

Anti-viral therapy is currently recommended, but it is unknown if current medications are effective for the treatment of EEHV; a number of animals treated with anti-viral medication have survived, and a number have also died. Factors indicating

whether an animal will survive or not have not been determined. A previous case study on the use of the anti-viral drug famciclovir in two cases of clinical EEHV determined that both elephants were viremic prior to treatment (based on PCR). Both elephants survived; the first case was given famciclovir orally and showed decreasing amounts of viral DNA in blood samples which also coincided with levels of famciclovir in serum. In the second case, famciclovir was administered rectally, decreasing amounts of viral DNA were observed - this may suggest an alternative route for administering antivirals to elephants (Schmitt et al., 2000).

The nucleoside analogue famciclovir is a prodrug which is then converted into penciclovir the active drug through phosphorylation by the viral thymidine kinase. Therefore, in order for nucleoside analogues to be effective, the virus must have a thymidine kinase (TK) gene. EEHV is the first betaherpesvirus discovered to have a TK gene, and there is evidence suggesting that the TK gene present in the EEHV genome is homologous to the one present in gammaherpesviruses. The TK found in EEHV is similar in structure to the TK found in HSV-1 (Ehlers et al., 2006). In addition to thymidine kinase, there is a serine/threonine PK gene in EEHV (Ehlers et al., 2006). This is a type of kinase

that possesses ganciclovir-phosphorylating activity. The results from this study suggest that the use of famciclovir in the treatment of clinical cases of EEHV may be effective due to the presence of viral TK; however, the presence of the PK gene would also suggest that other drugs such as ganciclovir, aciclovir and penciclovir may also be effective (Ehlers et al., 2006).

1.6.3.4.4 Diagnostic methods

Initial detection of herpesviruses in Asian elephants was achieved by electron microscopy and histopathology from necropsy samples (McCully et al., 1971). Inclusion bodies could been seen within the nuclei of capillary endothelial cells and sinusoidal cells (Ossent et al., 1990). To determine the type of herpesvirus present in Asian elephant samples, a PCR was developed to detect EEHV DNA. This was developed using EEHV terminase gene primers, and resulted in a 163bp PCR product from 7 deceased animals diagnosed with EEHV. However, this method only detected the presence of EEHV and did not determine which variant was present (Richman et al., 1999). A more sensitive and specific qPCR method was subsequently developed to determine sites of viral shedding in asymptomatic animals. This provided information on which samples can be tested to detect virus in both symptomatic and asymptomatic animals (Stanton et al., 2010). More recently, a

SYBR green qPCR assay was developed by Sariya et al. (2012) to test tissue from tongue, heart, kidney, spleen and liver samples. Samples which can be used for testing asymptomatic animals include oral-nasal secretions, conjunctival swabs, oral swabs and vaginal vestibule samples including swabs and biopsies. However, there is disagreement about which site provides the highest viral DNA yield; Hardman et al. (2011) suggested conjunctival swabs were more likely to yield viral DNA. Other studies have reported successful viral DNA detection from trunk washes (Stanton et al., 2010, Stanton et al., 2014).

Other methods have been developed to detect the host's immune response to EEHV. A recent study of 125 captive Asian elephants in the USA and Europe used an ELISA to detect IgG antibodies to EEHV in serum or plasma samples, multiple samples were used to ensure reproducibility (van den Doel et al., 2015). However, there are several disadvantages to this assay including the failure of this ELISA to detect certain antibodies conformational epitopes of gB, and a poor sensitivity due to high background. High background can be due to excessive colour development which leads to the obscuring of the true signal. Specificity testing was conducted using rabbit sera, a reaction with the antigen was not

observed. However, a positive response was observed when EEHV-gB specific sera were used, and this would suggest EEHV specificity.

1.6.3.4.5 Epidemiology

Most captive adult animals in the US and Europe that have been tested on a regular basis appear to intermittently shed EEHV. It is unknown if latency occurs, or if animals continue to actively shed the virus chronically. Evidence suggests that certain stressors may increase viral shedding similarly to other herpesviruses (Padgett et al., 1998, Freeman et al., 2007). In a study by Stanton et al. (2010), two pregnant animals were found to shed more often than other animals within the herd; an increase in the frequency of viral shedding during pregnancy was also suggested by Hardman et al. (2011). However, preliminary work conducted at The University of Nottingham suggested there is no link between Herpesvirus shedding and pregnancy in Asian elephants (Kenaghan, 2012).

Studies have concluded that most captive adult Asian elephants in the USA and Europe are infected with at least one EEHV subtype. A study in India found at least one EEHV variant present in 35% (16/46) of captive Asian elephants. EEHV5 was the most prevalent, and was detected in 20% (9/46) of trunk wash samples; EEHV 3/4 was in 9% (4/46),

EEHV1 in 7% (3/46), and EEHV2 and EEHV6 were not detected at all. In camp elephants, EEHV1 was the most prevalent at 14%, whereas in temple and orphanage elephants EEHV5 was more commonly seen at 37% and 20% respectively (Stanton et al., 2014). EEHV has also been found in wild elephants in India; it was suggested that EEHV was involved in the death of a two year old male calf (Press Trust Of India, 2014). Detection of EEHV1 has been confirmed in Laos, where a captive 2.5 year old male Asian elephant died, the only clinical sign observed before death was swelling the intermandibular region (Bouchard et al., 2014). It has also been confirmed in Cambodia, where a wild caught captive 3 year old female Asian elephant was found dead at a sanctuary, no clinical signs were observed (Reid et al., 2006). Lertwatcharasarakul et al. (2015) determined strains of EEHV in 8 samples from Asian elephants in Thailand; 75% (6/8) samples were positive for EEHV1 (of which 5 were EEHV1A and 1 sample was EEHV1B). Twenty-five percent (2/8) tested positive for the presence of EEHV4. It is currently unknown if the virus has an impact on wild populations.

EEHV has been detected intermittently in most captive European and North American asymptomatic adult animals. That have been tested using PCR to detect the virus, Hardman

et al. (2011) found a 100% prevalence of EEHV in a herd of 6 asymptomatic elephants in a UK collection, samples from a number of collection sites were used including trunk washes, oral swabs, conjunctival swabs and vulval swabs. Trunk washes are the sample most commonly used to detect the presence of EEHV in asymptomatic animals. A study of 5 Asian zoo elephants in the USA confirmed the presence of EEHV1 in 3 of the 5 elephants over a 15 week period (Stanton et al., 2010). By conducting longitudinal studies of virus shed from elephant populations, it can be determined if social and environmental stressors affect EEHV viral shedding. By contrast, the only published serology study, of 125 Asian elephants in USA and Europe, demonstrated 18% seropositivity and 19% were borderline for EEHV IgG antibodies (van den Doel et al., 2015). It is unclear how serum antibody titres vary with respect to viral latency, or whether antibody may vary relative to time since the last active infection.

1.6.3.4.6 Early detection and management

A number of suggestions have been made on how to manage elephant herds to improve early detection and treatment of EEHV. This includes daily checks by keepers: oral examination for ulcers and tongue lesions, monitoring of faecal bolus temperature and a general assessment of the animal. If a case

of EEHV is suspected, the current recommendation is that the attending veterinarian should complete a physical examination and collect a blood sample. If no other disease is diagnosed, treatment with famciclovir should commence. If the treatment is not successful, and the elephant dies, the veterinarian should submit post mortem tissues for further testing for EEHV (Lehman and Boehmer, 1999). Advice on the movement of captive elephants into a new collection includes: performance of an EEHV ELISA two weeks and one week before shipment, a PCR one week before, Packed Cell Volume /Complete Blood Count taken on the day of shipment along with a full medical examination. Risk periods should also be highlighted to keeping staff, such as pregnancy, weaning, transportation, introduction of new animals, social stressors, and sickness. It is currently unknown what/if risk periods are linked to development of symptomatic EEHV, or to viral shedding (Lehman and Boehmer, 1999). Attempting to control EEHV spread through preventative measures may not be possible as it is thought that the majority of the captive Asian elephant population are already infected with the virus.

1.6.3.4.7 Potential Co-infections in EEHV disease

There are a limited number of studies investigating co-infection with other pathogens in EEHV cases. It is not clear if the presence of co-pathogens affects the progression or

clinical signs of EEHV related haemorrhagic disease in clinical cases. A previous study investigated the potential presence of four other viruses which similarly target cardiovascular tissue, including: bluetongue virus (BTV), encephalomyocarditis virus (EMCV), haemorrhagic disease of deer, and equine arteritis virus. One animal tested positive for a co-infection, with both EEHV and EMCV being present in tissues (Richman et al., 1999). As there are a number of pathogens that can be transmitted to elephants in both wild and captive environments, many other pathogens could be investigated for their involvement in EEHV related deaths.

1.7 Final summary

The literature suggests that there is a current downward trend in the population of captive Asian elephants, therefore the sustainability of the captive population may still be questionable even with further research into EEHV. However, from the information reviewed there appears to be a number of gaps within the EEHV literature. It has been suggested that EEHV is the causative agent of a rapidly fatal haemorrhagic disease in Asian elephants, with the most fatalities occurring in juveniles aged below 5, though it is not understood why it mostly affects this age group. There is limited information within the literature on EEHV tissue tropism, further research

into this area would provide information as to where damage from EEHV infection may occur, which tissues contain the highest amount of EEHV, if EEHV is able to be present in a broad range of tissues and to determine which tissues support the growth of the virus.

There is a no research that has investigated the potential for a familial or genetic component to lead to an increased incidence of EEHV related death; this would provide further information on the possibility of EEHV being inherited, or if there is some inherited genetic susceptibility.

Currently there is no vaccine for the prevention of EEHV infection and there is limited information on the efficacy of anti-viral drugs. To enable further research into the efficacy of antiviral drugs, and for potential vaccine development, it will be necessary to develop a cell culture system which could be used to culture the virus, with Asian elephant cells the most likely to be able to grow the virus; at present, no such culture system exists for Asian elephants.

A limited number of studies have investigated the potential for co-infection with other pathogens in clinical EEHV cases, consequently it is unknown if co-pathogens may be involved in EEHV related deaths.

The aims and objectives for this PhD are therefore as follows:

1.8 Aims and objectives

This thesis contains a number of different studies with different aims and objectives:

Chapter 3:

The aim is to determine if there is a link between pregnancy and EEHV viral shedding, and this will be achieved by conducting the following objectives: 1. use qPCR to analyse trunk wash samples obtained over a 3 year period, 2. compare the data with glucocorticoid data to look at stress levels and 3. determine whether the presence of other potential stressors are present by looking at keeper records.

Chapter 4:

The aim is to culture endothelial cells from Asian elephant umbilical cord samples by conducting the following objectives: 1. isolate cells from umbilical cords obtained at birth from Asian elephants and 2. revive cells and attempt cell culture.

Chapter 5:

The aim is to determine the tissue tropism of EEHV1 in two Asian elephants whose deaths were associated with EEHV, and this will be achieved by conducting the following objective:

analyse and compare viral DNA loads from samples associated with EEHV deaths using qPCR.

Chapter 6:

The aim is to determine if there is a potential genetic or familial link between EEHV associated deaths, and this will be achieved by conducting the following objectives: 1. create a genogram of related Asian elephants located in North America and Europe, 2. calculate inbreeding coefficients and 3. conduct microsatellite analysis on samples obtained from Asian elephants in the UK.

Chapter 7:

The aim is to detect the potential presence of co-pathogens in EEHV infected Asian elephant samples using microarray technology, and this will be achieved by conducting the following objective: develop and optimise primers to detect EEHV and EMCV using microarray technology to determine the presence of co-pathogens.

2 Materials and methods

2.1 Ethical approval

Ethical approval for this project was granted by the School of Veterinary Medicine and Science, The University of Nottingham's non-animal scientific procedures act (ASPA) ethics committee. All samples were collected by trained keepers and staff at the establishment.

2.1.1 Elephant trunk wash collection and preparation

Fifty millilitres of sterile saline was poured into the nares of the elephant; the trunk was elevated for 20-30 seconds. The elephant was then instructed to blow the contents of the trunk into a bag. The fluid was then poured into a 60ml pot. This procedure is routinely used for TB testing. Samples were stored at 4°C or -20°C until transported to Nottingham on ice or ice packs. Samples were stored in -20°C freezer until processing.

Trunk washes were filtered to remove debris such as sand and hay. Non-woven gauze was attached to the bottom of a 25ml pipette; the sample was then strained through the gauze into a 50ml Falcon tube. The samples were then centrifuged for 10 minutes at 1,500xg. The supernatant was then removed, and the cell pellet was stored at -20°C until DNA extraction.

2.1.2 Post mortem tissue

Post mortem tissue was collected from five Asian elephants, the cause of death for four of the elephants was haemorrhagic disease, and EEHV was confirmed in these animals by qPCR. One of the elephants was negative for EEHV, and the cause of death is not determined. Samples were collected from each tissue using a borer and placed into a 7ml bijou tube. All samples were stored at -20°C until DNA extraction.

2.2 Nucleic acid extraction

2.2.1 Trunk wash and tissue DNA isolation

DNA was extracted using a Macherey-Nagel tissue kit following the manufacturer's instructions for buccal swabs and an extra step to elute highly pure DNA. To elute highly pure DNA the spin column was placed into a 1.5ml microcentrifuge tube, 50µl of preheated (70°C) buffer EB was applied to the column. Tubes were centrifuged for 1min at 11,000xg. A further 50µl of preheated buffer BE was added to the column and again centrifuged for a further 1min at 11,000xg. DNA extractions were stored at -20°C. All DNA extractions were conducted in carried out in a class II laminar flow hood to minimise contamination.

2.3 Samples used in this study

A number of different samples from Asian elephants have been used throughout this study including tissue samples from five elephants, trunk washes from nine elephants and faeces from seven elephants (table 9). The ID of the animals have been anonymised at the request of the collections.

Table 9: A list of samples used throughout this study. Asian elephants included have been anonymised.

ID	Samples used in this study
UON_1189	Kidney, tongue, lung, heart, liver, aorta, spleen, bone marrow, trunk lining and lymph node
UON_1329	Kidney, tongue, lung, heart, liver, aorta, spleen, pancreas, trunk lining and lymph node
UON_1002	Heart, liver and lung
UON_1490	Liver, lung, and kidney
UON_1149	Heart, liver, lung and kidney
UON_1140	Trunk wash
UON_1093	Trunk wash
UON_1625	Trunk wash
UON_1089	Trunk wash
UON_1175	Trunk wash
UON_1543	Trunk wash
UON_1579	Trunk wash
UON_1309	Trunk wash
UON_1353	Trunk wash
UON_1091	Faeces
UON_1285	Faeces
UON_1626	Faeces
UON_1027	Faeces
UON_1094	Faeces
UON_1405	Faeces
UON_1627	Faeces

2.4 Statistics

All statistics were performed using GraphPad version 7.01. Tissue samples were analysed and compared in chapter 5 using a one way ANOVA, and a Pearson product-moment correlation was used in chapter 7 to determine if there was a correlation in the presence of EEHV using PCR and microarray.

Chapter 3 – Longitudinal study of Asian elephants *Elephas maximus* indicates no link between Elephant Endotheliotropic Herpesvirus-1 shedding and pregnancy

3 Longitudinal study of Asian elephants *Elephas maximus* indicates no link between Elephant Endotheliotropic Herpesvirus-1 shedding and pregnancy

Please note this chapter includes the following publication: (Bennett et al., 2015)(Bennett et al., 2015)Bennett, Laura, et al. "Longitudinal study of Asian elephants, Elephas maximus, indicates intermittent shedding of elephant endotheliotropic herpesvirus 1 during pregnancy." Veterinary Record Open 2.1 (2015): e000088.

Text in *italics* indicates new text added which was not included in the original publication. Viral load has also been changed to virus DNA load throughout this chapter and EEHV-1 has been changed to EEHV1.

Author list and role:

Laura Bennett – Prepared the manuscript, analysis and conducted laboratory work

Stephen Dunham – Contributed to the manuscript and supervision

Lisa Yon – Contributed to the manuscript and supervision

Sarah Chapman – Sample collection

Megan Kenaghan – Laboratory work

Laura Purdie – Laboratory work

Rachael Tarlinton – Contributed to the manuscript, supervision and lead contact

3.1 Abstract

Introduction: EEHV1 is a viral infection of elephants that has been associated with a fatal haemorrhagic syndrome in Asian elephants. Previous studies have suggested that pregnant animals may shed more virus than non-pregnant animals.

Methods: This study examined whether pregnancy affected the frequency or magnitude of shedding of elephant endotheliotropic herpesvirus 1 (EEHV1) using Taq man real-time PCR on trunk washes from four female elephants from a UK collection over three time periods between 2011 and 2014. These periods included pregnancies in two animals (period 1 and period 3). Behavioural observations made by keepers were also assessed.

Results: During period 1 there was a high degree of social hierarchical instability which led to a hierarchy change, and was associated with aggressive behaviour. Also during period 1 EEHV1 shedding was of a higher magnitude and frequency than in the latter two time periods.

Conclusions: These results suggest that there is no clear relationship between shedding and pregnancy, and that

behavioural stressors may be related to an increase in EEHV1 shedding.

3.2 Introduction

Elephant endotheliotropic herpesvirus 1 (EEHV1) is a betaherpesvirus associated with a fatal haemorrhagic syndrome in juvenile Asian elephants (*Elephas maximus*) (Davison et al., 2009). Clinical EEHV disease can be very rapid, beginning with oedema of head and thoracic limbs, lethargy, anorexia and reluctance to drink, and progressing to ulceration and cyanosis of the tongue, and internal organ haemorrhage. Death can occur between one and seven days from onset of clinical signs (Ossent et al., 1990, Richman et al., 1999, Richman et al., 2000a, Richman et al., 2000b, Reid et al., 2006, Garner et al., 2009, Denk et al., 2012). More than 60 cases have been reported worldwide, with a mortality rate as high as 85 per cent (Latimer et al., 2011). There is no vaccine or other preventive measures for EEHV infection, but treatment with intensive care and famciclovir has been used, and in some cases, the animal has survived (Schmitt et al., 2000). This treatment is expensive: a course of famciclovir can be in excess of £10 000 per animal (US$17 000) (Redrobe, 2012). EEHV is currently not able to be cultured though qPCR of viral DNA in trunk washes, which are an established way of detecting EEHV viral shedding (Stanton et al., 2010). It has

recently been discovered through studies of zoo animals that many, perhaps most, captive adult Asian elephants intermittently excrete EEHV1 from oronasal mucosa (Stanton et al., 2010, Hardman et al., 2011), suggesting that subclinical infection is widespread. Viral DNA shedding can be detected using conjunctival swabs, buccal swabs, trunk swabs and trunk washes (Hardman et al., 2011, Stanton et al., 2013, Stanton et al., 2014). It is not clear why some animals develop fatal haemorrhagic disease and others only have latent or asymptomatic infections. The route of transmission of the virus is also currently unknown though transmission via nasal secretions from the trunk has been suggested (Stanton et al., 2010).

Herpesviruses exhibit both latent and lytic (active) phases. In the betaherpesvirus family, latency has been reported in lymphoreticular cells, secretory glands, kidneys and other tissues (Knipe and Howley, 2007). Reactivation of human herpesviruses from latency is thought to occur as a result of immunosuppression during stressful physiological states such as occurs in: pregnancy, organ transplant and chemotherapy patients (Sakamoto et al., 1982, Greenberg et al., 1987, Dahl et al., 1999). *In a study by Dahl et al. (1999), blood samples were taken from 104 pregnant women at 3, 5 or 6-8 months*

during pregnancy, and a blood sample was also taken at the time of birth. During pregnancy, HHV6 DNA was present in 158/416 (38%) of leukocyte samples collected from 104 women, which was significantly higher than at delivery in those same women, at which time 26/104 (25%) of the samples were positive, in comparison 5/21 (24%) of control samples tested positive for HHV6 DNA. This compares to 24% of women who tested positive for HHV6 in the control group. Sakamoto et al. (1982) investigated reactivation of the Epstein Barr virus in immune supressed pregnant women, the results suggested antibody titres were higher in those women in contrast to non-pregnant controls.

However, a reactivation during pregnancy is not needed to transmit herpes virus to the infant; indeed, it has been suggested that most cases of vertical transmission of herpes viruses (such as HSV2) during pregnancy, are transmitted during asymptomatic or unrecognized viral shedding episodes (Zuckerman, 2009). Transmission often occurs when a herpes infection is acquired during pregnancy, thus transmission to the infant may occur before symptoms occur from the primary infection or transmission occurs and the mother may remain asymptomatic (Brown et al., 1997, Whitley, 2004).

Many healthy non-immunosuppressed individuals continuously or sporadically shed various herpesviruses such as Epstein-Barr virus and herpes simplex virus 1 (Junker et al., 1991, Kaufman et al., 2005, Pierson et al., 2005). *In healthy individuals, stress is a known factor in the reactivation of herpesviruses, stress can occur in many forms including loneliness and examination stress. Using the loneliness scale developed by Bissinger et al. (2002), Glaser et al. (1985) investigated the stress of loneliness as a trigger. Virus capsid antigen antibody titres were used to determine the level of herpesvirus in each of the 30 participants, significant changes were observed between low and high loneliness groups. Those in the high loneliness group had higher antibody titres compared to those in the low loneliness group, higher titres were observed for EBV, HSV and HCMV, and however, changes were not seen in antibody titres for poliovirus type 2. Glaser et al. (1999) investigated the impact of final examination stress on military cadets in the USA, EBV antibody titres were higher in blood samples obtained during examination week in comparison to samples taken 6 weeks previously, significant changes were not observed in the antibody titres for HSV-1 or HHV-6. However, in contrast, a study of 308 immunocompetent adults, investigated potential stressors for herpes simplex virus eye infections. Potential*

stressors investigated included: wearing contact lenses, psychological stress, exposure to sunlight, eye injury, and menstrual periods. It was concluded that there was no evidence to suggest psychological stress and other stressors investigated were triggers of ocular HSV (Cohen et al., 2000).

Physiological measurement of stress levels in animals can be difficult; however, measurement of cortisol levels is usually accepted as an indicator of stress levels. Cortisol is a product of the hypothalamic–pituitary–adrenal axis, which is activated at times of physical or psychological stress (Foley et al., 2001, Möstl and Palme, 2002, Millspaugh et al., 2007). In many wildlife species, measurement of serum cortisol levels is impractical; obtaining blood from the animal can be stressful, and would interfere with the study as this handling could in itself increase cortisol levels. Faecal glucocorticoid metabolites (breakdown products of cortisol excreted in the faeces) are, therefore, used as a non-invasive measurement of stress in elephants (Millspaugh et al., 2007).

Several recent reports from longitudinal studies of EEHV shedding in zoo herds (Stanton et al., 2010, Hardman et al., 2011). Hardman et al. (2011) indicated that EEHV1 shedding detected by trunk washes may be increased during pregnancy; this trunk secretion could present a possible

transmission route to juveniles and neonates. Hardman et al. (2011) reported that pregnancy may affect the amount of EEHV virus shed; the pregnant animal in their study had a higher level of virus detected in trunk washes during the third trimester of pregnancy, compared with other stages of the pregnancy, or to the rest of the herd throughout the pregnancy. *Investigating EEHV during pregnancy is important as it is currently accepted that it is common for there to be an increase in herpes viral shedding, or a reactivation of the virus during pregnancy or birth in humans. A number of studies have been conducted on the reactivation of HSV2, especially in asymptomatic seropositive pregnant women. (Frenkel et al., 1993) suggested HSV2 is common among pregnant women. The study investigated antibodies (using western blot analysis) of pregnant women 439/1355 (32%) tested positively for HSV2. Infection was recognised as a first exposure based on antibody titres in 43/264 seropositive women during the study. This suggests there was a high amount of seropositivity due to a reactivation, the remaining 396 women had previously been infected with HSV2, and therefore a high prevalence of seropositivity was as a result of reactivation. The researchers noted that viral shedding occurred at a similar rate in both asymptomatic and symptomatic women. Co-infection with other viruses such as*

HIV has also been suggested to increase the chance of a herpesvirus reactivation during pregnancy. A study was conducted in which 60 HIV-positive and 8408 HIV-negative pregnant women were screened for genital herpes; 48/60 (75%) were found to be seropositive, in contrast to only 2709/8408 (32%) of HIV-negative pregnant women. The study further determined that 8% of HIV positive women and 2% of HIV negative women had a reactivation of herpes during labour (Hitti et al., 1997).

The current study is the first longitudinal cohort study of a group of four elephants that were studied over a period of three years. Samples were examined from 2011 to 2014 over three periods: during pregnancies in two animals that occurred at separate times (sample period 1 and sample period 3), and during a period when no animals were pregnant (sample period 2). Faecal glucocorticoid levels (for those time periods for which they were available) and behavioural records were also examined to analyse physiological or behavioural indicators of stress that may be linked with EEHV1 shedding. This permitted investigation of the suggestion that pregnancy may increase EEHV1 shedding frequency and magnitude (Stanton et al., 2010, Hardman et al., 2011). The current study used qPCR on trunk wash samples to examine EEHV1

shedding in an all-female closed herd at a European zoological collection.

3.3 Materials and methods

This study was approved by the School of Veterinary Medicine and Science (University of Nottingham) non Animals Scientific Procedures Act Committee, September 13, 2011.

Samples were collected from four female Asian elephants at a UK zoo. The elephants are referred to as elephant A, B, C and D. Samples were collected during two separate pregnancies, and one period where no animal was pregnant. Both pregnancies were the result of artificial insemination. Elephant hierarchical ranking was reported by the keepers, and was based on observations of dominant and submissive behaviours seen in interactions between the animals over time. A hierarchy change was observed on November 20, 2011 (Table 10).

Table 10: Elephants included in the study, showing keeper estimated rank, age and detailing pregnancies during three sample periods. Pregnancy status of each elephant is also shown, + = pregnant and - = not pregnant.

Elephant	Hierarchy rank before 20/11/2011	Hierarchy rank after 20/11/2011	Age	'Sample period one' (07/09/2011–26/03/2012) pregnancy status	'Sample period two' (27/03/2012-22/05/2012) pregnancy status	'Sample period three' (23/05/2012-20/03/2014) pregnancy status
A	2nd	3rd	16	-	-	-
B	1st	1st	29	-	-	-
C	4th	4th	30	+	-	-
D	3rd	2nd	19	-	-	+

Time period 1 (pregnancy 1) was from September 7, 2011 to March 26, 2012 (samples were taken for seven months during this period). Time period 2 (no animal pregnant) was from March 27, 2012 to May 22, 2012 (two months), and time period 3 (pregnancy 2) was from May 23, 2012 to March 20, 2014 (samples were taken for 11 months during this period). Sampling was not continuous during period 1 and period 2. One hundred and thirty-seven trunk washes were tested from the four animals over the three sample time periods. Trunk washes were collected by having the elephant draw up 50 ml of saline into its trunk, the trunk was then elevated for 30 seconds, and the elephant then forcefully expelled the saline into a plastic freezer bag. The contents were then transferred into a 60 ml plastic container, and were stored at −80°C until transport and processing of samples could be arranged. All

samples were treated alike. Before processing, trunk wash samples were thawed and filtered through gauze to remove sand and feed material, and were then centrifuged at 458 g for 10 minutes. The supernatant was discarded, and cell pellets were stored at −20°C until further processing.

DNA was extracted from trunk wash cell pellets, using a NucleoSpin Tissue Kit (Machery Nagel), as per manufacturer's instructions. To avoid cross-contamination, all work was conducted in a class II laminar flow hood, and a separate plastic opener was used for each 1.5 ml microcentrifuge tube.

Quantitative PCR analysis was conducted using a modified version of the protocol described by Stanton et al. (2010). However, alternatively sourced primers and probes and equipment brands were used, and the quencher dye on the probe was modified from MGB-NFQ to Eclipse.

EEHV viral shedding was normalised against interferon-γ (IFN-γ) using a standard curve; the qPCR cycle threshold value was converted into a copy number (the number of EEHV and IFN copies within each sample), and the number of EEHV copies was divided by the number of IFN-γ copies in each sample to obtain a ratio.

Freidman tests with Dunn's multiple comparison test as a post-hoc test, were performed in GraphPad Prism V.5.04 (GraphPad Software) for comparative virus DNA load (a)

between animals and (b) median virus DNA loads during each sampling period.

Faecal samples were opportunistically collected at irregular intervals from all animals. An aliquot of faeces was collected from the centre of the faecal bolus, and the material was manually broken apart and transferred into a 60 ml universal container. Faecal samples were stored at −20°C until analysis. Faecal glucocorticoid and progesterone analyses were performed using the protocol described by Watson et al. (2013).

Elephant keepers maintained records of the animal's behaviour; events recorded included submissive behaviour, aggressive behaviour, management changes, foot care and medical observations. Records were stored on the MedARKS database. Foot care events occurred at the same incidence throughout the study, and no major illnesses or injuries were sustained during the study period.

3.4 Results

During 'sample period 1' (pregnancy 1), all samples from all animals (61) tested positive for the presence of EEHV, with normalised virus DNA loads between 0.1 and 10.5. During 'sample period 2' (non-pregnant period), all samples (12) also tested positive for EEHV1 though at a lower magnitude of normalised virus DNA load (below 3.1). During 'sample period

3' (pregnancy 2), EEHV1 was detected in 8 out of 63 samples (12.69 per cent) though these samples had an even lower normalised virus DNA load of below 2.0 (Figure 10).

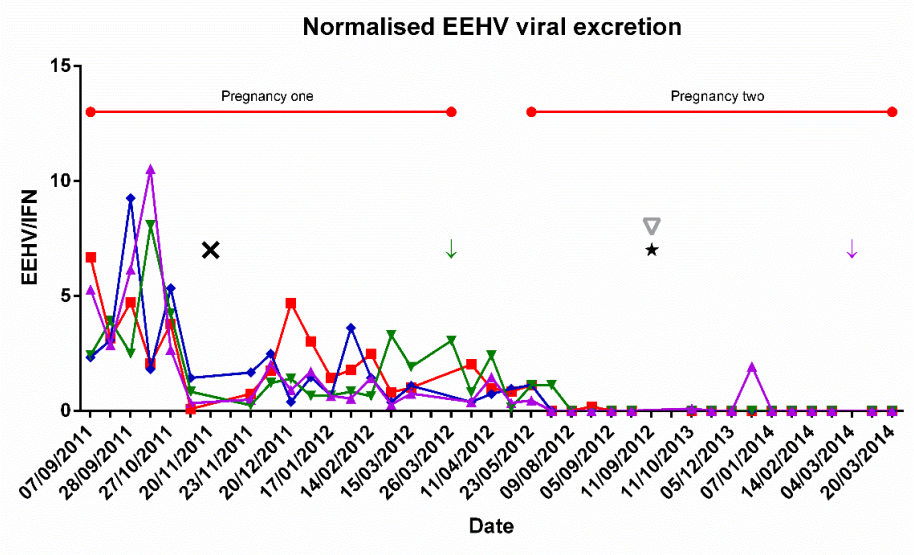


Figure 49: EEHV viral shedding in four female elephants, EEHV was normalised against IFNγ. Elephant endotheliotropic herpesvirus (EEHV) viral shedding in four female elephants. EEHV was normalised against interferon-γ (IFN-γ). Red squares=elephant A, blue diamonds=elephant B, green inverted triangles=elephant C, purple triangles=elephant D. Green arrow=elephant C gave birth, purple arrow=elephant D gave birth, black star=switched to protected contact, black X=change in hierarchy, grey inverted triangle=change in keeping staff and red horizontal bar=periods of pregnancy. The y axis is the amount of EEHV normalised against IFN-γ. EEHV was normalised against IFN-γ. EEHV copy number was divided by IFN-γ copy number to give a ratio. On the x axis is the date; note that dates were not continuous, as sampling periods were irregular.

From August 2011 until October 2011, before the change in hierarchy, elephants B, C and D showed elevated levels of EEHV. There was a drop in EEHV shedding in all animals in November 2011. This was then followed by a period of consistent shedding in all animals from December 2011 until June 2012. From August 2012 until March 2014, EEHV1 was detected in 81 out of a total of 137 samples (59.56 per cent) (Figure 10).

The median magnitude virus DNA load for each elephant was: elephant A, 1.28; elephant B, 1.17; elephant C, 1.18 and elephant D, 1.21. Elephant D had the highest frequency of positive samples at 61.7 per cent (22 out of 34). All samples were positive in sample periods 1 (pregnancy 1) and 2 (non-pregnant period). In sample period 3 (pregnancy 2), elephant D had the highest frequency of positive samples at 18.7 per cent (3 out of 16) (Table 11).

No significant differences were apparent in normalised virus DNA load between animals. However, median virus DNA load during time period 1 and 3 were significantly different (Freidman test P value 0.0046, Dunn's multiple comparison test time period 1 v time period 3 $P<0.05$).

Table 11: Percentage of positive samples for each elephant

Sample period	Elephant A	Elephant B	Elephant C	Elephant D
1	100%(15/15)	100%(15/15)	100%(16/16)	100%(15/15)
2	100%(3/3)	100%(3/3)	100%(3/3)	100%(3/3)
3	13.3% (2/15)	6.3%(1/16)	15.5%(2/16)	18.7%(3/16)
Total	60.6%(20/33)	55.8%(19/34)	60%(21/35)	61.7%(22/34)

A total of 50 incidents of aggression were recorded by keepers. During sample period 1, there were a total of 14 incidents. Over the five months of this period, this equated to an incident rate of 2.8 per month. During this time period,

elephant A suffered three injuries from aggressive behaviour from elephant D, and overall, injuries were most common during this sampling period. This is also the time during which the first pregnancy occurred. During sample period 2, 13 incidents of aggression were observed, and one injury was sustained by elephant A. The rate of incidents during this time equated to 6.5 incidents per month. During the third sample period, the second pregnancy occurred. There were a total of 23 incidents in this sampling period; the incident rate was 2.9 per month, with one incident causing an injury to elephant A (Figure 11). *Incidents involving more than one elephant were classed as 1 occasion, aggressive behaviour may also only involve a single animal where an animal exhibited aggressive behaviour, but other animals were not involved.*

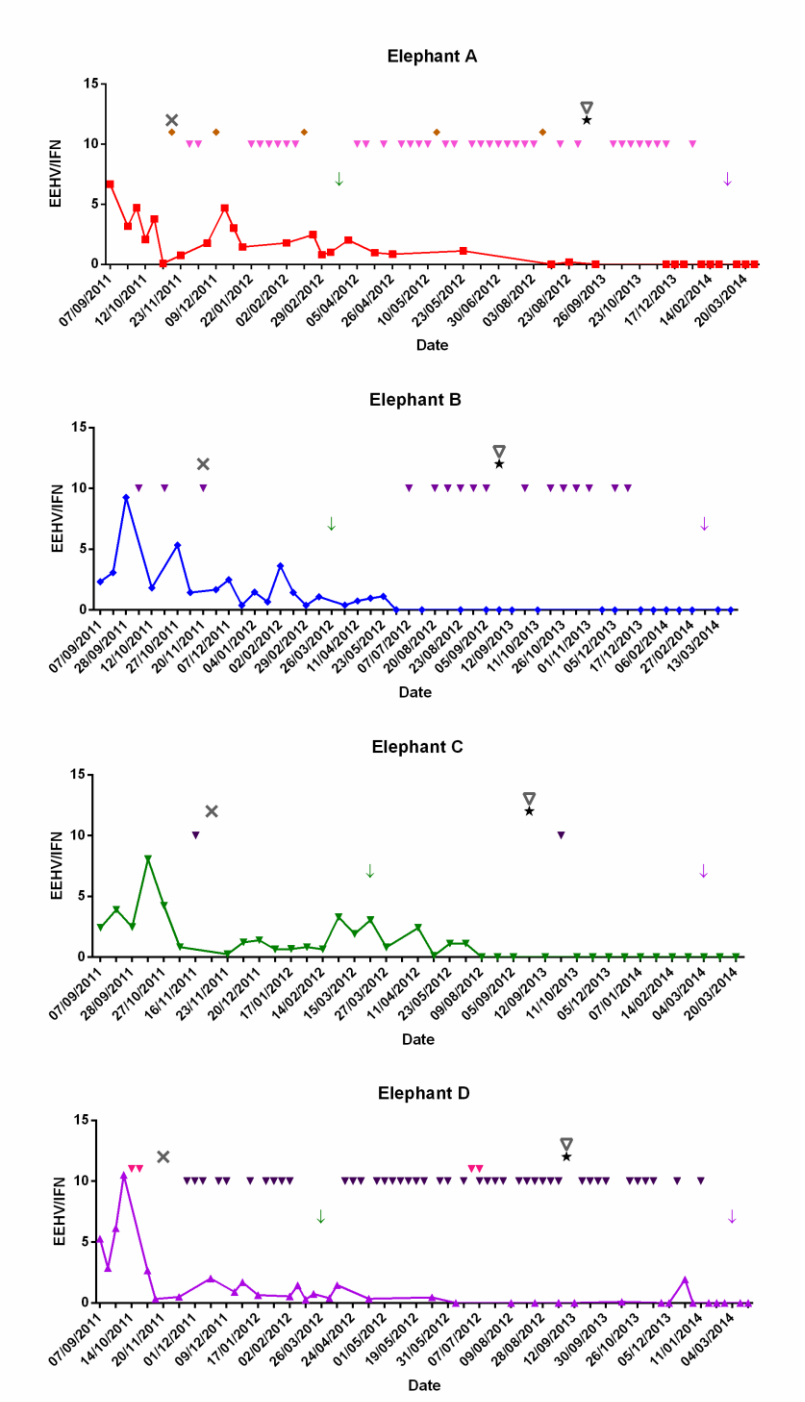


Figure 50: EEHV viral shedding and incidents of aggression in four female elephants. EEHV viral shedding and incidents of aggression in four female elephants. Red squares=elephant A, blue diamonds=elephant B, green inverted triangles=elephant C, purple triangles=elephant D, green arrow=elephant C gave birth, purple arrow=elephant D gave birth, black star=switched to protected contact, black X=change in hierarchy, grey inverted triangle=change in keeping staff, orange diamond=injury/reopened injury from aggression, purple inverted triangle=incidents of aggression/tension (aggressor) and pink inverted triangle=incidents of aggression/tension (victim).

Faecal glucocorticoids were monitored for short periods, which only partially overlapped with trunk wash sampling. No patterns of variation in glucocorticoid levels versus normalised virus DNA load were evident (Figure 12).

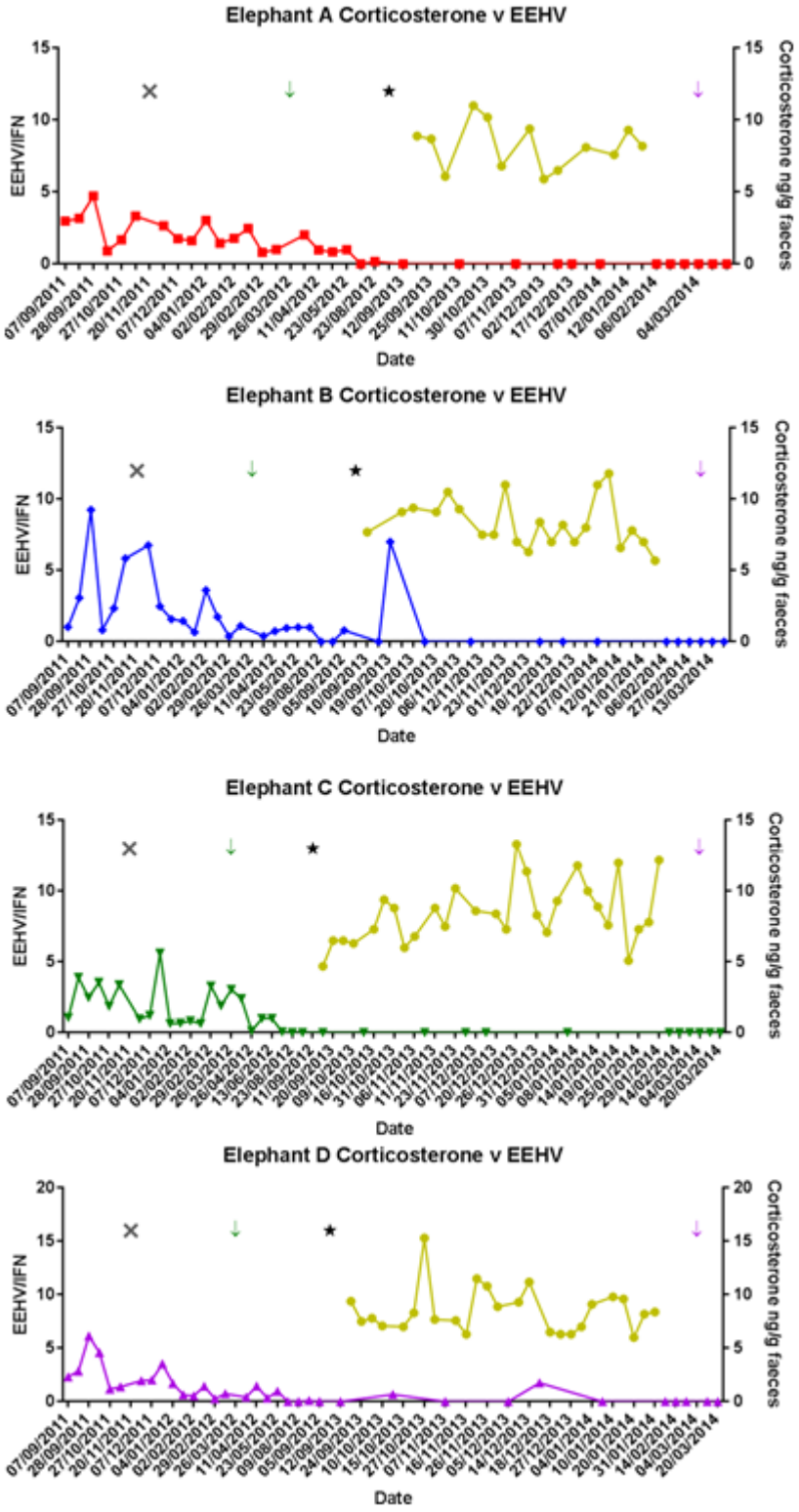


Figure 51: Glucocorticoid (corticosterone) levels versus normalised virus DNA load. *Corticosterone levels (round yellow points) from faeces were compared to EEHV virus DNA load levels (elephant A = red squares, elephant B = blue diamonds, elephant C = green inverted triangles and elephant D =purple triangles). Specific changes are also highlighted in each graph, switched to protected contact (black star), change in hierarchy (grey cross), elephant D gave birth (purple arrow) and elephant C gave birth (green arrow).*

A number of samples were Sanger sequenced (Source BioScience), and the presence of EEHV1 DNA was confirmed.

3.5 Discussion

In this study, EEHV was detected in four out of four elephants though results suggest that pregnancy did not result in increased EEHV1 shedding; indeed, median normalised load during the second pregnancy was significantly lower than during the first, which was not the case for the difference between normalised virus DNA loads for either pregnancy and the non-pregnant period in this study. Freeze thawing of the trunk wash samples may have resulted in some cell lysis and a reduced sensitivity of the present assay; however, all samples were positive for IFN DNA, indicating that cellular DNA was present, and have been presented as a ratio of viral to cellular DNA to compensate for variation in input DNA. During the first pregnancy, all four elephants shed EEHV1 consistently. During the second pregnancy, shedding was quite sporadic. Furthermore, during the period when no animal in the group was pregnant, shedding was continuous. This is in contrast to herpesvirus reactivation during human pregnancy, which has been reported for both human herpesvirus 6 and cytomegalovirus (Dahl et al., 1999).

In the current study, the study herd was closely monitored, and data were available for a number of other factors that might be linked to stress in the animals (and EEHV1 shedding). The faecal glucocorticoid results are difficult to interpret without previously defined baseline levels, but no clear patterns emerged between faecal glucocorticoid levels and EEHV1 shedding. However, because there were a limited number of samples used for hormone measurements during the sampling periods for EEHV1 testing, these results must be interpreted with caution. *All faecal glucocorticoid samples were taken post management change, so it was not possible to look at the effect this had. There are a number of limitations in using faecal glucocorticoids as a measure of stress, they may not be reliable as an animal can become used to a stressor and this may reduce the level of cortisol in faeces when exposed to that stressor (Möstl et al., 2002). There may be a link between diet and levels of glucocorticoids in faeces, as a diet that is high in fibre takes longer to pass through the gut which may result in less reabsorption of glucocorticoids. These factors may limit the reliability and the usefulness of using glucocorticoids as a measure of stress (Lewis et al., 1997, von der Ohe et al., 2004). There is limited information in the literature to determine if the levels in this study are reflective of the "normal" range.*

Keeper reports of behaviour were used to identify aggressive behaviours throughout the study period. Dominance-related aggression relating to hierarchy change has been observed in many other species, and incidents of aggression may occur for several days until a hierarchy is established (Houpt, 2011). Studies in other species have also indicated that corticosterone measures are more strongly linked with intensity of aggression than with frequency of incidents (Marsteller et al., 1980).

In this study, the highest level of EEHV shedding occurred during sample period 1 (pregnancy 1). This is the period during which keepers reported that a hierarchy change occurred on November 20, 2011. Elephant A also sustained an injury on this date due to aggressive behaviour. Analysis of the behavioural records showed that a large number of incidents involved elephant D as the aggressor and elephant A as the victim. This was not unexpected as these were the two elephants that the keepers reported had switched in their hierarchical ranking. Although there were not as many aggressive incidents during sample period 1 compared with other sample periods, the intensity appears to be greater as indicated by the higher number of injuries or reopened injuries that resulted during this period. This period of hierarchical

instability could have led to higher levels of stress in the herd, and suggests that EEHV shedding may be linked to behavioural stress in these animals.

It has been suggested that psychological or social stress is linked to the development of disease; this may be due to the onset of anxiety and depression due to the occurrence of stressful events. When stressful events occur they put the body into a negative state which affects biological processes, which in turn influences disease (Cohen et al., 2007). Social stress has also been suggested as a precursor to increases in asymptomatic shedding and the reactivation of herpesviruses; different stressors have also been shown to have different effects on reactivation. To investigate the relationship between social stress and the reactivation of HSV1, Padgett et al. (1998) investigated two potential stressors in mice: restraint, and social disruption. There was little evidence of HSV1 reactivation in mice that underwent restraint stress. By contrast, there was significant evidence that social disruption resulted in the reactivation of HSV1: In 15/37 (40.5%) mice, reactivation occurred in mice that experienced social disruption. Results also suggested that dominant mice were more likely to show a reactivation of HSV1; this may be because they were more likely to be involved in social conflict

and therefore experiencing more stress. A number of different psychological stressors have been shown to cause a reactivation of EBV in humans, including: experiencing spaceflights (for an astronaut) and undergoing academic examinations (Glaser et al., 1985, Glaser et al., 1999, Kennedy et al., 2000, Kim et al., 2002)

During the current study, elephant management practices at the facility also changed. Until September 11, 2012 (during sample period 2), the herd was managed using a 'free contact' approach (where keepers have direct physical contact with the animals). After this date, the management style changed to 'protected contact' (where there is always a physical barrier between the keeper and the elephant (Association of Zoos and Aquariums, 2011b). There was no clear connection between shedding and management methods, as frequency of viral shedding and magnitude of virus DNA load were already decreasing by this time, and decreased to negligible levels after these management changes. There is no information currently available on the impact of management changes and EEHV.

The results from this study suggest that that there is no clear relationship between shedding of EEHV1 in captive Asian

elephants and pregnancy. However, behavioural stressors may be related to an increase in EEHV1 shedding. It is also possible that EEHV shedding is affected by other, as yet unidentified factors, or that the animals may have been newly infected when the first sample set was taken. Further longitudinal studies on greater numbers of elephants, targeted monitoring of glucocorticoid levels, detailed behavioural observations and more detailed data on any behavioural indications of stress would add further support to the findings suggested by this study. However, the small sample size is a limitation of this study, this is due to the small herd at the establishment, and as such it is not possible to draw any conclusions as to the significance of the observations. Future longitudinal cohort studies would help to determine how EEHV shedding varies with time in individual animals and whether any increase in virus DNA loads are seen in associated pregnancies or parturition, or other factors such as management interventions.

3.6 Acknowledgments

The authors would like to thank Sophie Barnes (veterinary team) and the keepers at Twycross Zoo for collecting trunk wash samples and access to keeper logs, Dr Jeff Stanton for kindly providing plasmids containing EEHV1 MDP and Asian elephant IFN gamma (jstanton@bcm.edu; Baylor College of

Medicine, Texas, USA) and to Chester Zoo for access to faecal glucocorticoid data from the Twycross herd.

Chapter 4 – Asian elephant (Elephas maximus) endothelial cell culture from umbilical cord

4 Asian elephant (*Elephas maximus*) endothelial cell culture from umbilical cord

4.1 Introduction

The ability to culture cells to support a viral infection in vitro is important in establishing a laboratory grown viral stock. A cell culture system has not currently been established for EEHV, and viral isolation has also not yet been possible; this has resulted in difficulties in attempting to develop a vaccine or test drug efficacy.

Viral culture is a laboratory technique where samples of a particular virus are cultured within a cell type that they are able to infect. Visible changes in the cell morphology due to viral invasion may occur known as cytopathic effects (CPE) (Leland and Ginocchio, 2007). An example of herpesvirus CPE is the presence of inclusion bodies. Viral inclusion bodies can occur in liver cells infected with HSV, VZV and CMV. An example of an inclusion body are Cowdry type A bodies, which are composed of protein and nucleic acid, and can be seen using light microscopy (Tselis and Booss, 2014).

4.2 Use of viral cultures

4.2.1 Diagnostics

Viral cultures have been a traditional method of diagnosing disease, with the virus being isolated in the culture process. HSV is commonly detected in cell culture using primary rabbit kidney, mink lung or enzyme-linked virus inducible system (ELVIS) culture systems. Such systems are unable to detect VZV, but using a mixture of CV-1 and MRC-5 cells allows both HSV and VZV to be detected simultaneously (Huang et al., 2002b). However, using cell culture for diagnosis is slow, expensive and requires trained technicians. With the development of new technologies allowing for rapid detection of viruses, the use for viral culture in diagnostics has decreased. However, a large number of viruses have been propagated in cell cultures to allow for the development of vaccines, including small pox, yellow fever, flu and hepatitis A (Provost et al., 1986, Weltzin et al., 2003, Ehrlich et al., 2008).

4.2.2 Cell culture derived vaccines

Prior to development of cell culture methods, many viruses were propagated in embryonic chicken eggs. Advancing to cell based methods provides benefits such as the ability to produce vaccines rapidly during an outbreak, producing

vaccines which avoid egg based methods is important, especially as egg is a known allergen. Animal serum can also be avoided, as viruses can be grown in a synthetic media; this may prevent transmission of spongiform encephalopathies. There are also some disadvantages to this method: cells must be free from viruses, and virus yields may be low. Mammalian cell culture systems have been used to produce influenza vaccines, of which three are currently licensed by the Food and drug administration (FDA).

There are three types of inactivated vaccine: whole virus, split virus vaccine and subunit vaccine (Zuckerman, 2009). There are several stages in the preparation and production of a cell based vaccine: firstly, a high density cell culture of the appropriate cell type must be made; the cells are then infected with the virus. After viral propagation the culture is centrifuged and residual virus is removed. The virus is then inactivated; this may be done using several methods, including the use of solvents or detergents, pasteurisation or acidic pH inactivation. The virus can then be purified and used in a whole virus vaccine (Milián and Kamen, 2015). To produce a split vaccine, the virus particles are disrupted; to do this the inactivated virus is treated with detergents. Inoculation with a split virus produces fewer adverse reactions

than using whole virus (Zuckerman, 2009). The third type is the subunit virus vaccine; this type of vaccine stimulates an immune response by presenting the immune system with an antigen from the target virus. Hepatitis B vaccine RECOMBIVAX HB is an example of a subunit virus vaccine, the vaccine is made by cloning the Hepatitis B virus gene which codes for Hepatitis B antigens in yeast, the antigen can then be harvested from the cultures (MERCK, No date) For the influenza vaccine, for example, surface glycoproteins HA and NA are the antigens used, and the vaccine is purified by the removal of other viral components (Zuckerman, 2009, Milián and Kamen, 2015).

4.2.3 In vitro drug testing

In vitro cell cultures are used for initial testing on the efficacy of new drugs new compounds to treat viruses prior, to the use of animal models (in vivo). The testing of drugs for use in treating HSV in humans has mostly used rabbit and murine models (Brandt et al., 1992). There are advantages and disadvantages to both in vitro and in vivo methods. The main disadvantage using the in vitro method is that drug candidates that are successful in preventing viral replication in a laboratory setting do not always work when transferred to an in vivo trial (Rothman, 2002, De Clercq, 2005). Many drug candidates used for in vivo trials prove to be ineffective,

usually due to drug toxicity or failure to achieve therapeutic levels (De Clercq, 2005). However, there are advantages to using in vitro methods. It can be species specific (human cells can be used for drug candidates for human infections). In vitro methods are also automated and are simpler than in vivo methods, as they do not require the maintenance and care of live animals (Quignot et al., 2014).

4.2.4 Anti-herpetic drug candidate testing

When conducting drug testing there are welfare and ethical considerations to consider. Research conducted using endangered species presents with a number of other issues such as small sample sizes, which may limit the statistical robustness of the data (Hilton and Richardson, 2004). If living animals are used for essential experimental research, guidelines for animal welfare need to be followed. Workman et al. (2010) suggest that to ensure the welfare of the animals, the use of replacement, reduction and refinement should be incorporated into experiments; thus, researchers must ensure that other methods have been explored (replacement), the number of animals used remains as low as possible (reduction) and that the experiment is refined and optimised (refinement). Other studies have shown that to improve the welfare of test animals, environmental enrichment should be used as this led to an improved wellbeing. Enrichment is used

to create a more stimulating environment and can include toys in the environment, rodent wheels for mice, plastic tubes, gnawing blocks, and different nesting materials (Van Loo et al., 2002, Frick and Fernandez, 2003, Wolfer et al., 2004). Proxy species may be used in replacement for endangered species in research; however, a proxy species for either the African or Asian elephant has not yet been determined. There are limitations when using a proxy species that would need to be considered; for example, any results which are then trialled on the target species would need to be done so with caution, and proxy species may not be found for more unique animals

A number of studies have used both in vitro and in vivo methods to investigate drug candidates in the treatment of herpes infection. A previous study by Shigeta et al. (1983) investigated a number of anti-herpetic compounds to determine their inhibitory activity against VZV. Ten isolates of VZV (five clinical isolates and five laboratory strains) cultured in Human Embryo Fibroblast (HEF) cells were infected after three days of incubation. Three compounds were identified as potent VZV inhibitors. The study authors highlighted that to fully investigate the therapeutic potential of the compounds would require further investigation in an animal model (Shigeta et al., 1983). The need to find new drug candidates

has meant cell culture methods continue to be used. A more recent study by Kleymann et al. (2002) investigated the potential use of helicase-primase inhibitors as a new drug candidate in the treatment of HSV. This study however, tested the compounds on HSV-1 infected Vero cells. To investigate compounds further this study also included a murine challenge model. HSV-1 or HSV-2 was applied to the nares of female mice, they were then subsequently given an oral suspension of treatment compounds. The study concluded that the compound BAY 57-1293 both in vitro and in vivo, was found to be superior in the treatment of HSV in comparison to acyclovir, valaciclovir and famciclovir (Kleymann et al., 2002)

There is difficulty in using antivirals for the treatment of EEHV. Ultimately anti-viral drugs have not been tested in Asian elephants, therefore therapeutic levels are not known. The most appropriate and effective route of delivery is also unknown for antivirals in Asian elephants, however, levels of the drug in blood could be established with minimal invasion, this can be difficult in elephants as blood samples would need to be taken.

4.3 Cell lines used for the culture of herpesviruses

A number of cells have been used to culture herpesviruses including ovine fetal lung cells, mink lung cells, ferret lung cells, potoroo kidney cells, turkey embryo kidney cells, equine dermal cells, bovine turbinate cells, and porcine kidney cells (PK15); all were shown to be sensitive to a number of animal herpesviruses (Peterson and Goyal, 1988). Other cells such as marmoset B lymphoblastoid cells (B95-8), Vero cells, Human Fetal Lung Fibroblast cells (MRC-5), Cyprinus carp brain, human peripheral blood Mononuclear cells, guinea pig embryo cells, baby hamster cells, and rabbit kidney cells, have also been used (Landry et al., 1982, Gleaves et al., 1985, Shaw et al., 1987, Archer et al., 1999, Hukkanen et al., 1999).

4.4 Previous attempts at EEHV culture

Being able to culture EEHV would enable the potential development of a vaccine and the testing of anti-herpetic drugs for efficacy against the virus, as well as fundamental studies of the virus lifecycle and transmission route that are essential background information for the development of diagnostics, treatment and control strategies for the virus.

Several attempts have been made to isolate and culture EEHV in a variety of cell lines (Table 12). A previous attempt to

culture primary placental endothelial cells for an attempt at viral culture by Richman (2003) was unsuccessful.

Table 12: Previous attempts to culture EEHV in a variety of cell lines

Cell line	**Source**
Primary bovine lung cells	Metzler et al. (1990)
Chicken Embryo Fibroblasts	Metzler et al. (1990)
Madin Darby Bovine Kidney Cells (MDBK)	Metzler et al. (1990)
Rabbit Kidney 13 cells (RK13)	Metzler et al. (1990) and Richman et al. (1999)
Vero cells	Richman et al. (1999)
MARC African Green Monkey Kidney cells	Richman et al. (1999)
Embryonating chicken eggs	Richman et al. (1999)
Baby Hamster Kidney cells (BHK)	Richman et al. (1999)
Equine Epidermal cells	Richman et al. (1999)
Human Foreskin Fibroblasts	Richman et al. (1999)
Asian Elephant Fibroblasts	Richman et al. (1999)
African Elephant Fibroblasts	Richman et al. (1999)
Primary Placental Endothelial cells	Richman (2003)

4.5 Culture of endothelial cells

Primary cell cultures include fibroblasts, melanocytes and endothelial cells. Endothelial cells are derived from the endothelium of veins, commonly obtained from the umbilical vein, as this vessel is more easily available. Endothelial cells have been cultured to study blood pressure homeostasis and permeability. Human umbilical vein endothelial cells (HUVEC's) are frequently used as a model system, especially for the study of pathology and function of endothelial cells including physiological processes such as angiogenesis.

This study describes two methods used in attempts to culture Asian elephant endothelial cells. Endothelial cells were selected for culture for two reasons: they are undifferentiated cells, and EEHV has been previously been detected in haematoxylin and eosin stains in endothelial cells from clinical cases.

4.6 Materials and methods

Ethical approval for the procedures was granted by the School of Veterinary Medicine and Science, through their non-Animal Scientific Procedures Act (ASPA) ethics committee 13/09/2011. A total of seven attempts were made to culture endothelial cells; two methods were trialled during this study.

4.6.1 Method one

The first umbilical cord was collected on 12/10/2013 by elephant keepers and staff at a UK zoo. Small pieces of umbilical cord were washed twice in phosphate buffered saline (PBS) and transferred into pots containing Dulbecco's Modified Eagle Medium (DMEM). The DMEM contained 20% fetal calf serum, 5% Penicillin-Streptomycin, 5% Amphotericin B and 5% Fungizone. Cord blood was collected in a blood bag containing anticoagulants. Placenta and cord blood were transported to Nottingham at room temperature to minimise cell death.

4.6.2 Method two

Samples were derived from umbilical cord material from a natural birth following a normal 22 month gestation in a female Asian elephant in a UK zoo, which resulted in a live born female calf. The expelled placenta was collected, and a section of elephant umbilical cord was clamped. Artery clamps were attached to the free end of both umbilical arteries and the umbilical vein, and clamps were then attached 30cm further along the cord (Figure 13). The cord section was cut away from the remaining cord and placenta using a dissection knife. The cord section was washed once in lukewarm water to remove external debris, and a second time in phosphate buffered saline (PBS). The sample was then transferred into a sterile plastic transport box containing rinsing solution (1% penicillin/streptomycin, 1% heparin (138.8mg per 5ml PBS)) in PBS). Overalls, masks and gloves were worn by the investigators throughout sample handling.

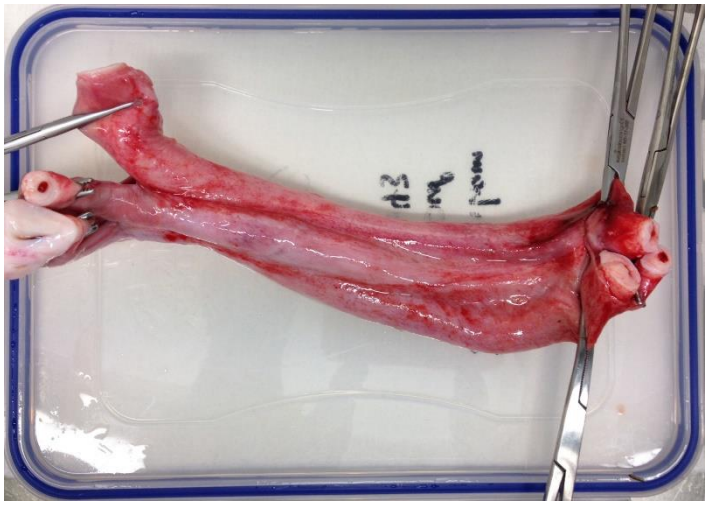


Figure 52: Asian elephant umbilical cord prior to separation of both arteries and the umbilical vein. Arteries and the umbilical vein were clamped using artery clamps.

Cord processing was conducted in a laminar flow hood to minimise contamination. Both arteries and umbilical vein were separated using a surgical blade (Figure 14).

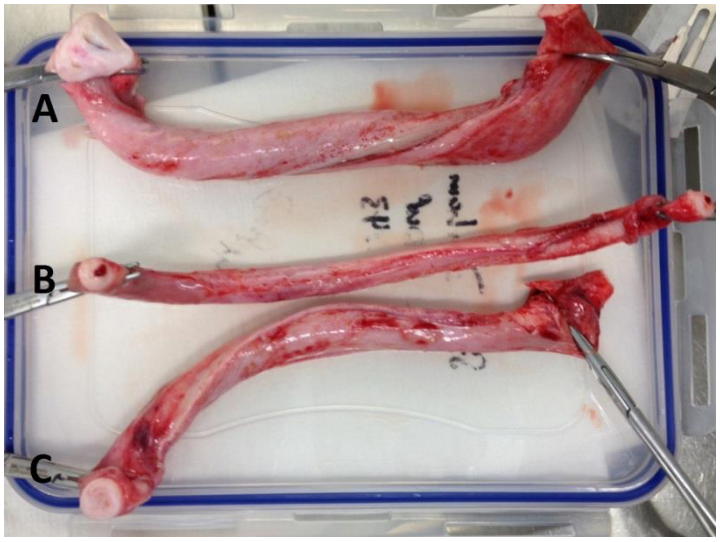


Figure 53: Elephant umbilical cord, artery and vein separation. A = artery, B = umbilical vein and C = artery. Both arteries and the umbilical vein were separated from each other using a surgical blade. Clamps remained attached to minimise external contamination.

Blood was washed from the vessels with phosphate buffered saline (PBS) through a 4-French canine urinary catheter. Twelve millilitres of pre-warmed collagenase (Sigma-Aldrich,

Dorset, UK) at a concentration of 1mg/1ml was injected into each artery and vein, and the sample was incubated for 15 minutes at 37°C. Twenty millilitres of endothelial media [10% foetal calf serum, 0.01% human Epidermal Growth Factor (hEGF) (BD biosciences, Bedford UK) in 400ml human endothelial serum free media (Life technologies (Thermo Fischer), UK)) was used to wash cells from each artery and vein into a 50ml centrifuge tube and to inactivate collagenase. The harvested cell suspension was centrifuged at 484xg (Thermo Scientific Sorvall Legend RT centrifuge) for 5 minutes; the supernatant was then discarded, and the cell pellet resuspended in endothelial media.

Cell culture flasks were coated in fibronectin (0.1% fibronectin in RPMI media), and the cell suspension was added and incubated at 37°C and 1% CO_2 until cells were 50-70% confluent. Media was changed every other day and cells were passaged twice a week.

4.6.3 Immunohistochemistry

Cultured cells were transferred onto microscope slides using cytospin (All cytospins in this thesis were performed using a Thermo Shandon cytospin 4 machine); cells were first suspended in 100µl human endothelial media, 100µl of the cell suspension and 50µl foetal calf serum were put into each

cytospin funnel. Samples were spun at 500rpm for 5 min and air dried for 30 minutes. Cells were fixed using acetone, wrapped in foil and stored at -20°C overnight and then transferred to -80°C until further processing. The cells were then stained using a protocol for visualising cells, using von Willebrand factor (VWF) as a biomarker for endothelial cells (Robinson et al., 2006). The primary antibody used was polyclonal rabbit anti-human VWF (Dako, High Wycombe, UK) diluted in PBS with 2% Normal Goat Serum. Normal rabbit IgG diluted in 2% normal goat serum was used for negative controls, and the secondary antibody was biotinylated goat anti-rabbit IgG (Vector Labs, Peterborough, UK). Primary antibodies were detected using Vectorstain ABC and visualisation was performed using diaminobenzidine (Vector Labs) as described by Robinson et al. (2006).

Aliquots of cultured cells from each passage were stored in freezing media (Lonza, Slough, UK) and in liquid nitrogen for future use.

4.6.4 DNA barcoding

DNA was extracted from the cultured cells using a commercial kit following the manufacturer's instructions for extracting DNA from cultured cells (Machery Nagel Tissue kit). Cells used

for DNA extraction were at a concentration of $20x10^6$/ml, of which 100µl was used.

DNA barcoding primers were used to confirm the origin of the cells. Primers were as follows: forward primer C 5'-TTTCAACCAACCACAAAGACATCGG-3' and reverse 5'-TATACTTCAGGGTGTCCAAAGAATCA-3' (Bitanyi et al., 2011). The sample was amplified using PCR; this included 35 cycles of 94°C for 30 secs, 55°C for 30 secs and 72°C for 1 min (Bitanyi et al., 2011). DNA bands were visualised using gel electrophoresis and Nancy 520 fluorescent stain, and the PCR product was then sent to Eurofins for Sanger sequencing using the same primers to confirm the identity of the product.

4.7 Results

4.7.1 Method one

Two attempts were made to isolate endothelial cells; method one was unsuccessful, as only fibroblast like cells were present in both cultures. The cells derived from attempt one survived until passage 3, and attempt two until passage 1; at this time both became contaminated with bacteria and yeast.

4.7.2 Method two

A total of five attempts were made using method two. Attempt 3 using method two was successful, and endothelial cells were

present; this will be discussed in more detail below. Attempts 5, 6 and 7 all became contaminated on the 4th, 2nd and 3rd day respectively. Three umbilical cord samples were between 4-6 days old on arrival; the cord used for attempt one was three days old and the one for attempt two was 2 days old. The umbilical cord used for attempt 3 was processed within 2 hours from the time of parturition, and attempt 7 was within 10 hours (Table 13).

Table 13: A brief account of each EUVEC attempt.

	Method	Age of cord	Survival time	Cell growth
Attempt 1	One	A few days	To passage 3	Fibroblast like cells
Attempt 2	One	2 days	To passage 1	Fibroblast like cells
Attempt 3	Two	Within 2 hours	To passage 8	Endothelial cells present
Attempt 4	Two	6 days	Contamination on day 2	No significant growth
Attempt 5	Two	4 days	Contamination on day 4	No significant growth
Attempt 6	Two	5 days	Contamination on day 2	No significant growth
Attempt 7	Two	Within 10 hours	Contamination on day 3	No significant growth

Cultured cells from attempt three survived until passage 8, and they appeared to be morphologically similar to endothelial cells with a "cobblestone" effect (Figure 15). After passage 2 there began to be a mixed population of cell types.

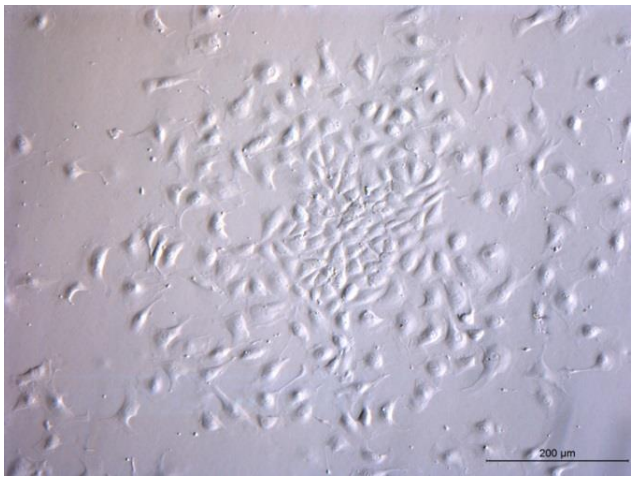


Figure 54: Cultured cells at passage 2 40x resolution. Cultured cells appeared morphologically similar to endothelial cells with a "cobblestone" effect.

The cells were stained for the presence of VWF to determine cell type, VWF was chosen as a biomarker as VWF lines the internal surface of both bone marrow cells and blood vessels, VWF is made within endothelial cells. Cultured cells from passage 2 stained positively for VWF (Figure 16).

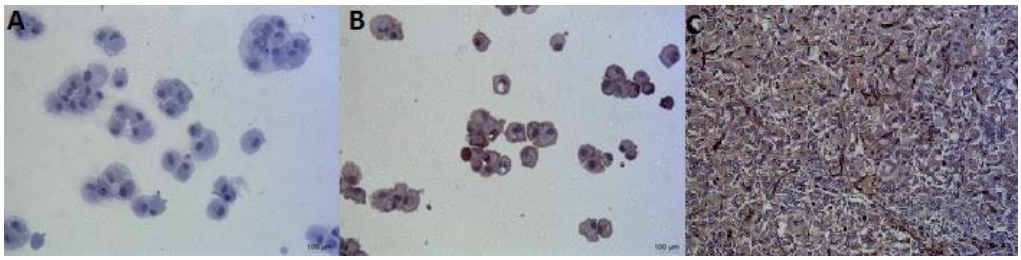


Figure 55: Visualisation of passage 2 endothelial cells using von Willebrand Factor as a biomarker on cultured cell cytospin. A= passage 2 elephant cells negative control, B = positive elephant endothelial cells showing the presence of von Willebrand Factor and C = positive control bovine corpus luteum. Cells were stained using a protocol for visualising cells, using von Willebrand factor (VWF) as a biomarker for endothelial cells.

Cells from passage 6 also stained positively for VWF, but the negative control for this passage was also mildly positive (Figure 17). By passage 6 cells appeared less morphologically like endothelial cells.

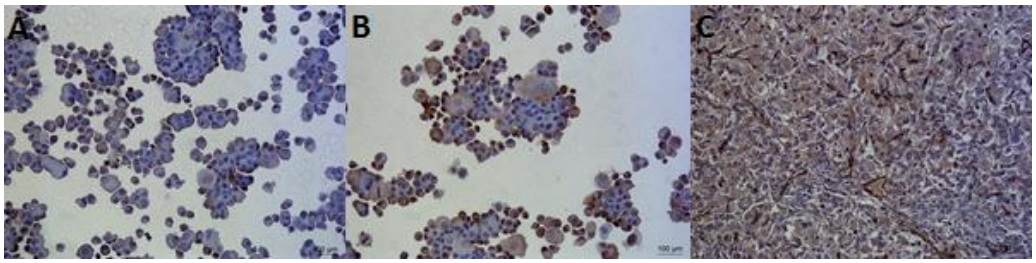


Figure 56: Visualisation of passage 6 cultured cells using von Willebrand Factor as a biomarker on cultured cell cytospin. A= passage 6 elephant cells negative control, B = positive elephant endothelial cells showing the presence of von Willebrand Factor and C = positive control bovine corpus luteum.

Sequencing and DNA barcoding results confirmed that the cultured cells were of Asian elephant origin (sequence not

shown) and the sequence was deposited on Genbank (KP233819). Comparing the sequence obtained from the cultured cells to the mitochondrial complete genome of each species, the sequence was 99% identical to *Elephas maximus*, 95% identical to *Mammuthus primigenius*, 95% identical to *Loxodonta cylotis* and 94% identical to *Loxodonta africana*.

4.8 Discussion

This study demonstrated the successful culture of elephant endothelial cells obtained from an Asian elephant umbilical cord. The cells cultured survived to passage 8; the passage 1 cell population appeared to be predominantly endothelial cells, while from passage 2 onwards there appeared to be a mixed cell population. This is similar to results from previous unpublished attempts by other researchers (Paul Ling, personal communication).

Endothelial cells may play a role in EEHV pathogenesis; this is suggested by the presence of haemorrhages observed in various tissues in acute cases. Endothelial cell dysfunction has been related to viral haemorrhages, as viral infection of the endothelium can alter the function of endothelial cells, such as their ability to control permeability resulting in vascular leakage. The endothelium also controls platelet interactions;

during viral infection of the endothelium, platelets may become aggregated or sequestered which can also result in thrombocytopenia (Krishnamurti et al., 2002, Carr et al., 2003, Dewi et al., 2004, Chen et al., 2007). However, herpes viruses such as cytomegalovirus are thought to infect endothelial cells; this could play a key role in the spread of HCMV throughout the body. HCMV may therefore infect endothelial cells without causing vascular leakage or thrombocytopenia. Endothelial cells are also thought to play a role in HCMV latency (Ho et al., 1984, Grefte et al., 1993, Jarvis and Nelson, 2002, Jarvis and Nelson, 2007). Similarly, it has been suggested that endothelial cells may also serve as the site of EEHV latency (Seilern-Moy et al., 2016). A recent study suggested that due to the large number of basophilic intranuclear inclusion bodies in Asian elephant deaths associated with EEHV, endothelial cells located in the endocardium and the myocardial arteries were more severely affected. This was compared to those in the: kidney, lung, liver, skeletal muscle and bladder (Seilern-Moy et al., 2015). Despite the successful outcome of this study, several further attempts to culture elephant endothelial cells from other umbilical cord samples were not successful. An attempt was also made to revive cell stocks from liquid nitrogen; this was unsuccessful. There are a number of challenges to performing

this type of culture. One of the key considerations is the age of the umbilical cord; efforts to isolate EUVECs from umbilical cords which were collected more than 4 hours after parturition were unsuccessful. Contamination is another common problem; this type of culture is highly prone to contamination with environmental contaminants such as bacteria and yeast. One of the other considerations in conducting this type of work is the difficulty in obtaining Asian elephant umbilical cords; as pregnancies do not occur frequently in captive elephants, the availability is limited.

There are also a number of other challenges in obtaining Asian elephant umbilical cords such as, animals may be kept in protected contact conditions this may make it difficult for keeping staff to enter the enclosure. If the animals are kept in free contact conditions, it may be dangerous to enter the enclosure following a recent birth. The animals may also consume or damage the umbilical cord prior to retrieval. Due to potential difficulties in retrieval, the umbilical cord sample may be in the enclosure for several hours before it is collected and prepared for shipping, thus collection conditions are not always known. However, in future attempts to culture EEHV, other sources of elephant endothelial cells may be used; endothelial cells are also found in bone marrow and

blood vessels (Shi et al., 1998, Crosby et al., 2000). It would however, be difficult to obtain bone marrow or blood vessels from a living Asian elephant. Samples could be obtained after death, but post mortem examinations usually take place several days after the elephant has died. Samples obtained after several days would not necessarily be viable for cell culture as prompt sample collection is important to allow successful cell culture. Samples could be obtained after death, but post mortem examinations often take place several days after the elephant has died. This may be due to a number of reasons, including the size of the animal, availability of suitable transportation, specialist staff availability and whether the death was expected or sudden.

The first steps in the development of a vaccine for EEHV or testing antivirals are to develop the ability to culture the correct cell type (susceptible to infection with EEHV), isolate EEHV, and attempt a viral culture. However, there are a number of factors that need to be considered when attempting to develop vaccines or test drugs in endangered animals. There are difficulties in the ethics of treatment; both drug and vaccine trials would require experiments on Asian elephants, as there is no animal model for this species. It would also be difficult to perform both vaccine and drug tests in the natural

host, as Asian elephants are a protected species. The testing of drugs in a sick animal would also be difficult, or even the possibility of not treating with anti-virals to determine if the animal would survive without treatment, as not providing any form of treatment may be determined as unethical.
It is the responsibility of the zoo to provide care for all animals and to provide treatment for any clinically ill animals and is covered under the Zoo Licensing Act 1981 (DEFRA, 2012).

BIAZA state that one of their main priorities is the promotion of zoo animal welfare, and that the needs of the animals must be met; this includes medical treatment (BIAZA, No date).

In the UK every zoo is required to have a license under the Zoo Licensing Act 1981, mistreatment or not providing good care to zoo animals could result in the revocation of the establishment's license, an example of this is South Lakes Zoo in the UK, whose owner had the renewal of their zoo license refused due to the deaths of 486 animals within four years (BBC News, 2017).

In the case of EEHV, treatment has included treating with anti-viral drugs of unknown efficacy in the Asian elephant. Attempting treatment of EEHV in Asian elephants can be seen

as justifiable as zoos are required to treat a sick animal to try and improve their health, even if there has been no evidence to prove the medication is successful. However, this may also be seen as inappropriate as zoos may be driven to treat EEHV in Asian elephants with drugs not proven to improve their health due to legal/ethical requirements. As zoos are not currently releasing Asian elephants into the wild and contributing to the survival of wild populations it may be suggested that keeping and treating animals in captivity is not ethically justified. But whilst Asian elephants are in captivity in the UK, zoos are required by law to provide care and treatment for medical conditions. However, developing a vaccine for EEHV may not be justifiable for a number of reasons including cost, unknown potential side effects and it is not known if it will have an effect on increasing the numbers of captive elephants. It is also unknown if a vaccine is developed how or if it would be possible to vaccinate wild Asian elephants and what impact this would have on wild populations if any.

Future work should build on the results from this study; this could include further attempts to revive endothelial cells from storage and attempting an EEHV infection. Cell to cell transmission could be investigated using infected post mortem

tissue placed onto cells in cell culture. The identification of other cells present within the population of mixed cells after passage 3, could be determined using other antibodies which have been developed for the identification and detection of endothelial cells. A number of antibodies (cell markers) have been developed in other species; however, their use in elephant studies is currently unknown. A number of existing antibodies which could be tested include the mouse monoclonal (RECA-1) to endothelial cells (anti-endothelial cell antibody), which has been shown to react with cells in the vascular endothelium; it has been successfully used in rat endothelial cell studies (Duijvestijn et al., 1992, Kang et al., 2001). Mouse monoclonal (BW-200) to endothelial cell anti-endothelial cell antibody, which reacts with the endothelium of the renal glomerulus in humans could also be tried (Abcam, n.d). Testing cell markers that have reactivity for a wide number of species could also be attempted. For example, VEGF R3, a mouse monoclonal antibody, has been developed for the identification of endothelial cells; VEGFR-3 mRNA is expressed in most endothelial cells, such as those found in the placenta, brain, intestine and lung. VEGF R3 antibody is reactive in a number of animals including bovines and canines (Neuromics, n.d-b). EMAP II antibody has shown reactivity in a large number of species, including cattle, cats, chickens,

horses, primates, rats and rabbits. It is a tumour derived cytokine that inhibits endothelial proliferation and can potentially induce apoptosis (Neuromics, n.d-a). This list, however, is not an exhaustive list as there are a large number of antibodies available; one of the major limitations in testing a large number of antibodies is cost.

If attempting a culture of cells for use in EEHV experimentation, another possibility would be to investigate the potential use of endothelial cells from other species such as horses or humans. Using elephant EGF or elephant foetal calf serum could be tested; this may aid in the growth of cells, and may provide more natural conditions for cell growth. A viral infection could also be attempted in other cell types.

Chapter 5 – Tissue tropism of EEHV1 and clinical disease

5 Tissue tropism of EEHV1 and clinical disease

5.1 Introduction

Tissue tropism can be determined using the measurement of viral DNA or RNA present (viral load) in tissues; viral load can also be measured in blood, or other body fluids, and can be used to diagnose or monitor the progression of clinical disease. A number of previous studies have investigated the correlation between disease severity and higher virus DNA loads for a number of different viruses. Zink et al (1999) concluded that the severity of Simian Immunodeficiency Virus encephalitis was linked with higher viral loads in both the brain and cerebral spinal fluid. Higher levels of HSV2 viral loads in the autonomic ganglia have also been correlated with an increased severity in clinical disease (Weinberg et al., 2003). Similar results were also found by Malavige et al. (2008), who investigated the relationship between viral load and disease severity in primary infections of VZV. Blood samples were obtained from 34 patients with varicella infection. The severity of disease was determined by the presence of fever, and the number and character of lesions. Viral load was measured for each patient using qPCR. Those patients who had more than 500 lesions had higher viral loads when compared to those with less than 500 lesions. The findings suggested patients with a mild infection presented

with lower viral loads than those with moderate to severe infections (Malavige et al., 2008).

One of the current problems facing the management of captive elephants is the prediction of which animals might develop disease due to EEHV. A number of previous studies have measured virus DNA loads in asymptomatic animals using trunk washes, it remains unclear whether levels of viral DNA present in trunk washes can be used to predict or even diagnose clinical disease.

Virus DNA loads have also been measured in the blood of Asian elephants. In an animal determined to be clinically ill, a virus DNA load of 1,464 viral genome copies was present per ml of whole blood 2 days before the onset of clinical signs. A blood sample was also taken from the same animal on day 8 of clinical signs, and showed an increase in the virus DNA load, which peaked at 635,992 viral genomes/ml blood (Stanton et al., 2013). Another previous study reported the range of viral genomes per ml of blood at $6.4 \times 10^5 - 2.6 \times 10^7$, however, elephants' whose deaths were associated with EEHV1 presented with higher virus DNA loads. This would suggest higher amounts were present in the animals that are clinically ill. However, it is difficult to be certain, as samples

were not obtained from the elephants before they became clinically ill, and a virus DNA load range was not determined for "healthy" elephants. Using blood to detect EEHV in asymptomatic animals is not recommended, as obtaining blood samples from 'healthy' asymptomatic animals can be difficult and is invasive. Another consideration is that blood samples are only recommended if the elephant is in the viremic stage where EEHV can be detected in blood (Hardman et al., 2011). Whereas trunk washes are less invasive, and have successfully been used to determine asymptomatic virus DNA loads (Stanton et al., 2010), however, trunk washes may be less useful during active infections when a blood sample may provide more valuable information. When EEHV is present in blood it is considered a clinically active infection, and higher levels of the virus are present in blood.

The presence of EEHV1 has been detected using a variety of methods including histology, serology and conventional PCR.

5.2 EEHV detection and presence of inclusion bodies

Basic methods to detect EEHV include using histopathology to examine post mortem samples. This includes looking for the presence of inclusion bodies, which are an indication of infection with a herpes type virus. Inclusion bodies are aggregates of proteins in cells, which are usually sites of viral

transcription and replication (Augenbraun et al., 1995, Jarvis and Nelson, 2002, Lahaye et al., 2009, Hoenen et al., 2012). A number of diseases are diagnosed on the basis of inclusion bodies, such as lewy body inclusions in Parkinson's disease and neuronal inclusion bodies in frontotemporal dementia (Gibb and Lees, 1989, Englund et al., 1994, McKeith et al., 1996, Gelb et al., 1999, McKeith et al., 2005). There are a number of different types of inclusion bodies currently identified for different diseases; some inclusion bodies form in the cytoplasm and others in the nucleus. These include: intracytoplasmic eosinophilic, intranuclear acidophilic and intranuclear basophilic (Table 14).

Table 14: Viral inclusion bodies and virus association

Inclusion body type	**Inclusion body and association**	**Reference**
Intracytoplasmic eosinophilic	Negri bodies (Rabies)	(Gupta and Varshney, 2000)
	Guarnieri bodies (Vaccinia)	(McCouch et al., 1997)
	Paschen bodies (Variola/small pox)	(Clayton et al., 1995)
	Bollinger bodies (Fowlpox)	(Cambon-Thomsen, 2004)
	Henderson-Patterson bodies (Molluscum contagiosum)	(McGuire and Beskow, 2010)
Intranuclear acidophilic	Cowdry type A (HSV/VZV)	(Parsons et al., 2005, Bayes et al., 2000)
	Torres bodies (Yellow fever)	(Bowkett et al., 2009)
	Cowdry type B (Adenovirus)	(Kierepka et al., 2016)
Intranuclear basophilic	Cowdry type B (Adenovirus)	(Kierepka et al., 2016)
	Owl's eye appearance inclusion bodies (CMV)	(Stanton et al., 2016)
Intranuclear and intracytoplasmic	Warthin-Finkeldey bodies (Measles)	(Brown et al., 2007)

Inclusion bodies have been found in proliferative cutaneous lesions of captive African elephants in the USA. Jacobson et al. (1986) investigated elephants from two groups: 33 animals from an animal importer (group one), and group two which were 63 elephants collected in Zimbabwe. Animals in both groups exhibited lesions; seven animals from group one had fibrous growths on their trunk which were present on arrival,

whereas fibrous growths did not appear on the trunks of animals in group two until three months after arrival. Biopsies from the lesions were taken at various stages, which showed younger nodules were inverted papilloma's, whereas older lesions were composed of fibroblasts, collagen and mixed cells. Electron microscopy also confirmed the presence of inclusions in the centre of early lesions. Lesion samples showed mature virus particles in the cytoplasm and in the intercellular space. The study concluded that the cutaneous papilloma lesions were associated with a herpes like infection.

Inclusion bodies have also been found in studies of EEHV; Richman et al. (2000a), found lesions in a number of target organs (including heart, liver and tongue), and the capillary endothelial cells also contained amphophilic to basophilic viral inclusion bodies. Further investigation of the inclusion bodies by electron microscopy revealed viral capsids of 80-92nm in diameter which were consistent with the morphology of herpesviruses. Similar results were suggested by Sripiboon et al. (2013), who noted the presence of cowdry type A inclusion bodies in epithelial cells within heart tissue samples from an EEHV associated death, this was determined by the use of haematoxylin and eosin staining.

The first discovery of a herpes like virus in African elephants was in 1971. For histology, lung samples were stained with haematoxylin and eosin, and for electron microscopy thin sections were stained with uranyl acetate. Lymphoid nodules associated with cowdry type A inclusion bodies were observed in the lungs of 37/50 (74%) wild African elephants, while inclusion bodies were found in both epithelial and syncytial cells, limited information was given about elephants such as gender, age etc. (McCully et al., 1971). Twenty-eight years later a PCR based assay was then developed to determine the presence of EEHV; however, this assay could not detect specific variants and was not quantitative (Richman et al., 1999).

5.3 EEHV tropism

The name 'elephant endotheliotropic herpes virus' suggests the virus has a tropism for endothelial cells; this is similar to HCMV which also has endothelial cell tropism (Jarvis and Nelson, 2007). Indeed, it appears that EEHV has a primary tropism for endothelial cells, especially those of the heart, liver and tongue (Brock et al., 2012).

However, it has been suggested that EEHV has a broader tropism than previously suggested, as it has been associated with both mild mucosal lesions and more severe haemorrhagic

disease (Schaftenaar et al., 2010). Tissue tropism of EEHV can vary case by case, as was observed by Sripiboon et al. (2013); in one of two fatal cases attributed to EEHV infection, tissue tropism was more specific, with mainly the cardiovascular system being affected, whereas in the other case, haemorrhages were noted in most organs. Tissue tropism also appears to differ depending on the variant of EEHV present; in fatal cases involving EEHV1, the cardiovascular system appears to be more severely affected. However, there appears to be less selective organ tropism for EEHV3, 4 and 5 (Garner et al., 2009, Sripiboon et al., 2013). Infection with EEHV3 has been associated with retinal damage and renal medullary haemorrhages; EEHV3 also appears to have a tropism for larger veins and arteries (Garner et al., 2009). Sripiboon et al. (2013) noted EEHV4 infection appears to be linked with multiple organ haemorrhages, including respiratory, cardiovascular and gastrointestinal systems. It was also noted that EEHV4 appears to have less selective organ tropism in comparison to other EEHV variants. In a case of EEHV5, widespread haemorrhages and oedema were found (Wilkie et al., 2013).

5.4 EEHV virus DNA loads

A number of previous EEHV studies have used qPCR to detect viral DNA; those developed have either used probe based or SYBR green technology. The first probe based qPCR for specifically detecting EEHV1 was developed in 2010 by Stanton et al. (2010). The PCR was used to detect EEHV1 in oronasal secretions of five asymptomatic animals; the assay detected up to 1,200 copies/ml in whole blood. The detection method, which was designed to detect a segment of the U38 (POL) gene, incorporated a separate qPCR assay for cellular IFNγ DNA in order to confirm the presence of cellular DNA in the samples and absence of PCR inhibitory factors. Subsequently, several probe based qPCR assays were described which were developed to specifically amplify EEHV 2, 3, 4, 5 and 6. However, discrimination between EEHV 3 and 4, was not possible as primers specific to each variant could not be developed (Lischka and Zimmermann, 2008). A SYBR green based qPCR assay has also been developed to detect EEHV1 from trunk swabs, with the primers based on the terminase gene sequences of EEHV 1-4. The assay detected low levels of EEHV1 DNA (276 copies/µl). The primers used for this assay were not tested on other sub types of EEHV (2-6), as positive control material was not available. The disadvantages of using a SYBR green assay is that non-

specific binding of primers may occur (resulting in false positives), and the PCR reaction may be inhibited by some dyes (Smith, 2010). The advantage of this method is that it is quantitative; the number of viral copies present in a sample can be calculated. In this method, virus DNA load is measured against a standard curve. A standard curve is a quantitative graph; it is created using a serial dilution of multiple samples, of which the properties such as the number of molecules is known. These samples are known as standards, the number of molecules in each dilution can then be calculated using Avogadro's number. Avogadro's number is the number of molecules in one mole of a substance. The sample used for a standard curve is usually a dilution series of an appropriate cytokine such as interferon or tumour necrosis factor alpha (TNFα), or beta actin. It is then transformed into a plasmid, or genomic DNA (from a host tissue sample) can also be used. The standards are run alongside the unknown samples in a qPCR reaction; the cycle threshold value (the cycle number at which the fluorescence generated crosses the fluorescence threshold) of the unknown samples which can then be compared to that of the known standards by interpolation on the graph.

Understanding the amount of virus present in both asymptomatic and symptomatic animals is important in the study of EEHV. The level at which an asymptomatic case becomes a clinical case is currently unknown. Therefore, further investigation into tissue tropism, and determining levels of the virus in tissues from animals whose deaths are associated with EEHV, would provide further evidence of how much virus is present in symptomatic animals. This study used a probe based qPCR assay to measure the virus DNA load in different Asian elephant tissues, obtained from two deaths associated with EEHV.

5.5 Materials and methods

Ethical approval for the procedures was granted by the School of Veterinary Medicine and Science, through their non-Animal Scientific Procedures Act (ASPA) ethics committee 13/09/2011.

5.5.1 Samples

Ten post mortem tissue samples were received for each of the two juvenile Asian elephants who presented with clinical signs consistent with EEHV disease; their deaths were attributed to haemorrhagic disease. The juveniles in this study included a two-year-old female who died on 03/07/2013 and a 3-year-old

male who died on 29/07/2013. All tissues had been stored at -20°C prior to arrival were stored at -20°C until processing.

5.5.2 DNA extraction

See materials and methods section 2.2.1

5.5.3 DNA content

The amount of DNA in each sample was determined using a nanodrop machine (Thermo Fischer nanodrop 8000) (see Table 15).

Table 15: Samples analysed for the presence of EEHV1 and the amount of DNA present.

UON_1189	DNA ng/µl	UON_1329	DNA ng/µl
Kidney	147.6	Kidney	35.76
Tongue	40.6	Tongue	24.59
Lung	176.3	Lung	14.17
Heart	39.7	Heart	235.6
Liver	181.4	Liver	76.3
Aorta	136.6	Aorta	19.73
Spleen	136.7	Spleen	77.9
Bone marrow	277.1	Pancreas	456.1
Trunk lining	169.6	Trunk lining	25.99
Lymph node	254.3	Lymph node	300

5.5.4 Quantification of EEHV1 by qPCR

To confirm the presence of EEHV1 by qPCR, the method described by (Bennett et al., 2015) was used, which was an adapted protocol based on the method described by Stanton et al. (2010). EEHV primers were used to amplify the U38 (POL) region; the forward primer was fluorescently labelled with FAM. Primers amplifying IFNγ were used to create a

standard curve, and the forward primer was also labelled with the fluorescent dye HEX. Both set of primers were included in each qPCR reaction.

5.5.5 Data analysis

Data was analysed using GraphPad Prism 7.01 software.

5.6 Results

5.6.1 Quality control of tissue DNA samples

In order to confirm that DNA samples extracted from elephant tissues contained DNA of sufficient quality to detect EEHV1, a qPCR to detect elephant IFNγ was performed on each of the samples. The presence of DNA of sufficient quality and quantity allows robust PCR amplification. The cycle threshold (Ct) value obtained for IFNγ was then used to normalise the qPCR data for EEHV (Figure 18). All samples tested gave a Ct value of between 18 and 22; a lower Ct value indicates a higher amount of elephant DNA within the sample. There was little variation between the IFNγ present in the samples. This would indicate fluctuations in the amount of EEHV1 was not due to variation in IFNγ.

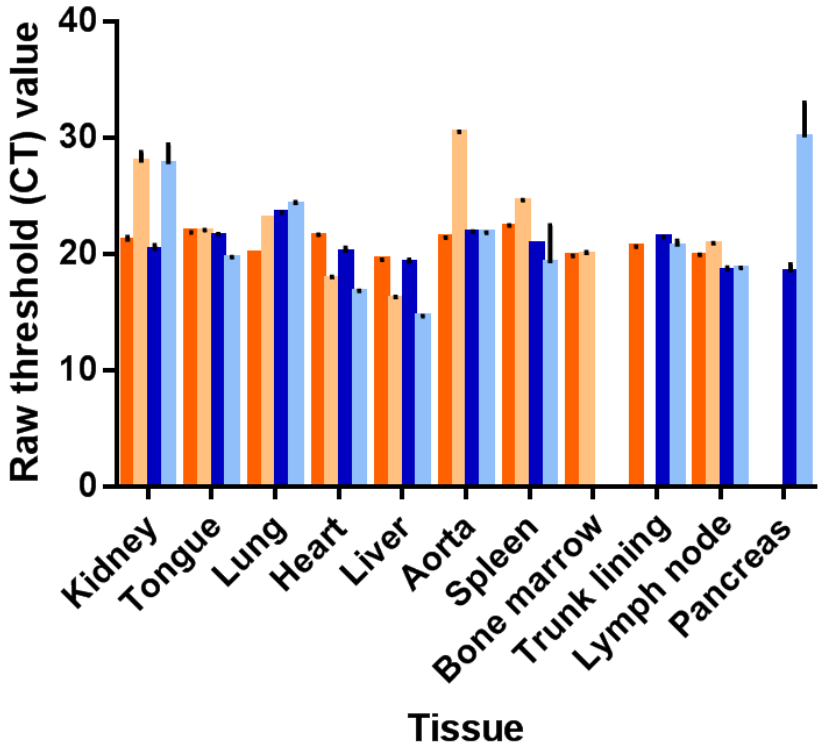


Figure 57: Comparison of mean raw threshold (CT) values and error bars. Raw IFNγ values for UON_1329 are dark orange and EEHV1 values are in light orange, raw IFNγ values for UON_1189 are in dark blue and EEHV1 values in light blue. Black bars above each column show the standard of error most bars showed a small standard of error.

5.6.2 EEHV1 presence in tissues

Ten tissues from UON_1189 and ten from UON_1329 were used in this study, of which 75% (15/20) were positive for EEHV viral DNA. Of the ten tissues provided from UON_1189, 70% (7/10) were positive; 80% (8/10) of 10 tissues from UON_1329 were positive.

5.6.3 UON_1189

EEHV viral DNA was present in almost all samples provided for this animal except the trunk lining; however, the amount of viral DNA present in each tissue varied considerably. The

tissues containing the highest amounts of viral DNA were the heart and liver, there was 10 fold more virus in the heart than in the liver. This was also confirmed using a one way ANOVA, the result when comparing the amount of EEHV in the heart when compared with the liver was statistically significant with a P value of 0.008. Lower amounts were found in the tongue and spleen, and the lowest amounts were present in the kidney, lung, aorta, bone marrow and lymph node. By contrast, no viral DNA was present in the trunk lining sample (Figure 19).

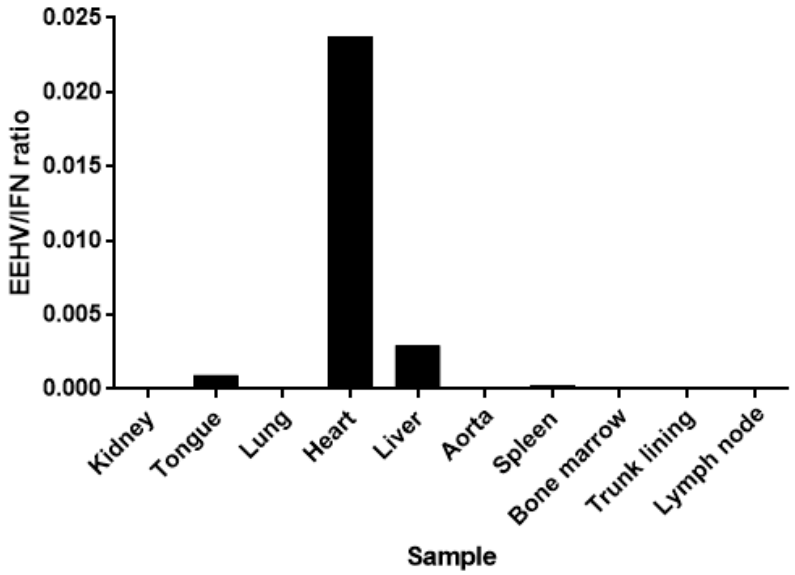


Figure 58: Ten samples from UON_1189 were tested for the presence of EEHV1, samples were normalised against IFNγ, and are displayed as a ratio of EEHV/IFNγ on the y axis. The highest levels of EEHV1 were present in the heart and liver, whilst lower levels were found in the tongue, bone marrow, lymph node and lung. The lowest concentrations were present in the kidney and lung, whilst no EEHV1 viral DNA was present in the kidney, aorta or the trunk lining.

5.6.4 UON_1329

EEHV1 viral DNA was present in eight of the samples from this animal; 80% (8/10) of the tested samples were positive. Varying levels of viral DNA were also observed between the tissues examined. The highest viral DNA loads were present in the liver and heart, there was 3 fold more virus in the liver than in the heart, however, analysis using a one way ANOVA showed that the results were not significant with a p value of 0.4. Lower amounts were seen in the tongue, trunk lining, lung, aorta and spleen. EEHV1 viral DNA was detected in all samples; however, only very small amounts were detected in the kidney, pancreas and lymph node (Figure 20).

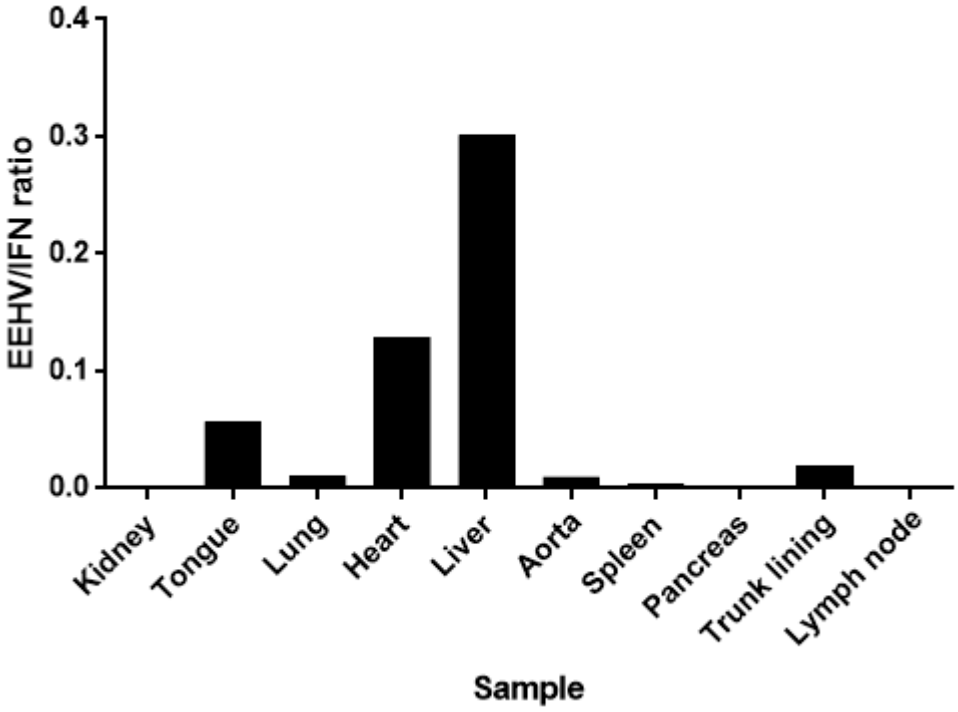


Figure 59: Ten samples from UON_1329 were tested for the presence of EEHV1, samples were normalised against IFNγ, and are displayed as a ratio of EEHV/IFNγ on the y axis. The highest concentrations of EEHV1 were present in the liver and heart, whilst lower concentrations were found in the tongue, trunk lining, aorta and lung. The lowest concentrations were present in the kidney and pancreas, whilst no EEHV1 viral DNA was present in both the kidney and pancreas.

5.7 Discussion

There are a limited number of studies within the literature that investigate EEHV tissue tropism and virus DNA load. To investigate the presence of EEHV1 viral DNA in samples from two deaths associated with EEHV1, qPCR was used. This method was chosen for this study as it allows the quantification of viral DNA in each tissue sample, the detection of smaller quantities of viral DNA, increased sensitivity, and requires a lower amount of starting material, in comparison to conventional PCR.

The tissues which contained the highest level of EEHV1 differed between the two animals in this study; the heart sample from UON_1189 and liver sample from UON_1329 appeared to have a higher viral copy number in comparison to other tissue samples obtained. In contrast, a study by Seilern-Moy et al. (2015) found the highest levels of EEHV4 in the lymph node and lowest in the heart, whereas for EEHV1A, the highest levels were in the lymph node and heart and the lowest were in the lung. In the Seilern-Moy et al. (2015) study EEHV1A and 4 were detected using a qPCR developed to detect EEHV1, 3, 4 and 5. Other viruses, such as hepatitis B virus, are known to have a tropism for liver (Sung and Lai, 2002); herpesviruses are also known to damage both the

heart and liver, this has been reported in neonatal EBV infection, where a post mortem showed the most damage occurred in the heart and liver (Avgil and Ornoy, 2006). The findings of the current study, are also similar to, the multi tissue tropism of HCMV fatal congenital inclusion disease, where a large number of different tissues have shown to be positively infected including; brain, placenta, heart, pancreas, liver, spleen, lung, bone marrow and small bowel. However, significant infections were found in the lungs, pancreas and kidneys which differed from the findings in the current study (Bissinger et al., 2002)

Clinical viral DNA/RNA load levels have been investigated especially in those presenting with a clinical infection. A study investigating recurrences of HSV1 and HSV2 and viral DNA load in 22 patients, the peak load of viral DNA was 123.6 viral genomes per cell (Boivin et al., 2006). A more recent study determined the level of HSV viral DNA in the cerebrospinal fluid samples of 29 patients with herpes simplex encephalitis. Viral DNA loads were between 2×10^2 and 42×10^6 (Schloss et al., 2009). Mathematical models have also been used to predict herpesvirus transmission, a study by (Schiffer et al., 2014) used a mathematical model to predict at what level HSV2 transmission is more likely to occur. It was predicted

that HSV2 was more likely to occur at viral DNA load levels greater than 10^4 viral genomes.

In studies of deaths associated with EEHV, higher levels than these have been observed. Latimer et al. (2011) found levels of EEHV3/4 at 10^7 viral copies per ml. The authors of the study also suggested that as the levels of EEHV3/4 were high, they were likely to be the primary viral agent responsible for haemorrhagic disease in the two Asian elephants studied.

Overall the results suggest that EEHV can be present in a wide variety of tissues. However, there were a number of limitations in this study. Only two animals were studied, as tissues from clinical cases of EEHV are difficult to obtain, so a limited number of animals is common in the studies of EEHV. In this study, only the presence of EEHV1 was determined and no other sub types.

Future work investigating virus DNA loads in connection with EEHV could provide more information on the levels of the virus during clinical disease and during asymptomatic periods. However, this would need samples to be taken during an asymptomatic period, and again if the animal becomes clinically ill. This would also require the same type of sample

being taken, allowing for a comparison to be made. Understanding the levels of virus DNA load may potentially enable elephants to be monitored, and enable determination of the appropriate time to administer prophylactic treatment when the virus DNA load reaches a pre-determined threshold.

Chapter 6 – A preliminary study into a potential genetic or familial link with EEHV associated deaths

6 A preliminary study into a potential genetic or familial link with EEHV associated deaths

6.1 Introduction

There is no current published research exploring the possibility of a familial link with EEHV deaths. Most herpesviruses cannot be inherited; however recent research has shown that Human Herpesvirus 6 (HHV6), is able to integrate into human chromosomes. A mother carrying HHV6 DNA integrated at chromosome 22 and a father carrying HHV6 DNA at chromosome 14 passed this genetically to their daughter, who had HHV6 DNA at both locations identical to her parents; this would suggest that HHV6 can be inherited (Daibata et al., 1999). It is unknown if any other herpesviruses, especially those of animals, can be transmitted in this way. A link between genetics and EEHV may also be possible through an inherited susceptibility to infection. Determining if there is the possibility of a familial link between EEHV associated deaths can be investigated in a number of ways: firstly, looking at relationships between the affected animals, and then looking at their genetic diversity.

6.1.1 Types of genetic testing to determine parentage

Genetic testing can be used to determine the parentage of a human or animal; this can be then used to compare levels of relatedness and inbreeding between one population and another. There are a number of methods that have been used to determine parentage.

6.1.1.1 Blood typing/serology

The first type of paternity testing was the use of blood types in humans; this method compared the specific antigens present in the blood which determine the blood type of the child and the parent. The blood typing system ABO is the most common; parentage can be determined as A and B alleles are co-dominant and O is recessive; a child with blood group O cannot have a father with AB blood. This method does not directly determine if a man has fathered a specific child, it only indicates if someone with a specific blood group fathered the child. Using the RH, kell and duffy blood grouping antigen system has made this type of testing more effective due to increased accuracy (Krøjgaard et al., 2009).

6.1.1.2 Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) was one of the first DNA profiling techniques developed (Murnaghan, 2016). The process begins with a sample of DNA, which is digested into fragments using restriction enzymes. The different sized restriction fragments are then visualised using gel electrophoresis, and the sample is then transferred to a membrane and further analysed using a Southern blot procedure. A labelled DNA probe is then hybridised to the membrane; this procedure then determines the length of the fragments which are complementary to the probe (Beckmann and Soller, 1986, Williams et al., 1990, Nieminen et al., 2011). Each fragment is considered to be an allele; an RFLP occurs when variation in the length of the fragment is observed between individuals.

RFLP has applications in genetic linkage, gene mapping and the localization of genes in the study of genetic disorders (Williams et al., 1990, Hyten et al., 2010, Ku et al., 2010, Wang et al., 2010, John et al., 2012). This technique has a number of limitations, as it requires a large DNA sample, it is a slow procedure, costly, non-automated, technically

demanding and there are low levels of polymorphism in some species (Cornell University, 2003, Cheriyedath, 2016).

6.1.1.3 Microsatellites

Genetic diversity can be measured using microsatellites (simple sequence repeats), which occur in non-coding DNA regions. They are short segments of DNA which have a repeated sequence. Repeats can be di, tri or tetra nucleotide, the number of the repeats can be highly variable, this is why they are useful as genetic markers. A genetic marker is sequence of DNA which can be used to identify individuals as its location on the chromosome is a known factor. This type of genetic analysis has been used for a number of species, including humans. A genetic map of the human genome based on 5,264 microsatellites was completed in 1996 (Dib et al., 1996).

Microsatellites have been used extensively in parentage testing in a number of species. Schnabel et al. (2000) validated 15 markers for use in parentage testing of American bison (*Bison bison*). Markers were chosen from the USDA cattle mapping database, and a number of criteria needed to be met for a marker to be selected, which included: suitability for multiplex PCR, high heterozygosity, large number of alleles, lack of known null alleles and allele size range. Of the

15 loci tested, the study found that five had a greater number of alleles in bison compared to domestic cattle (Schnabel et al., 2000). Parentage testing can be invaluable in certain breeding and domestic animal industries. For example, Bowling et al. (1997) determined the parentage of 4903 quarter horses using both blood typing and microsatellites; fifteen loci of blood group and protein polymorphisms (blood typing) and 11 dinucleotide microsatellites were studied. The validation of the techniques showed an effectiveness of 98.2% for microsatellite testing and 97.3% for the blood typing method.

There may also be evidence for the use of microsatellites across other species; for example, human microsatellites may also be applicable for investigating genetic variation in apes and old world monkeys. Coote and Bruford (1996) selected 85 microsatellites for determining paternity in apes and old world monkeys, the use of markers was previously published during the human genome mapping project. The results showed 11 markers were polymorphic across nearly all the species investigated, including: De Brazza's monkey (*Cercopithecus neglectus*), Diana monkey (*Cercopithecus diana*), black and white colobus (C*olobus spp*), langur (*Semnopithecus spp*), Sulawesi macaque (*Macaca nigra*), baboon (*Papio spp*),

Guinea baboon (*Papio papio*), mandrill (*Mandrillus sphinx*), orangutan (*Pongo spp*), gorilla (*Gorilla spp*), chimpanzee (*Pan spp*) and human. A more recent study also found human microsatellite markers effective in a study producing a genetic linkage map of the baboon (*Papio hamadryas*) (Rogers et al., 2000).

6.1.2 Samples for genetic testing

For genetic testing the most commonly used sample is blood or tissue however, these can be difficult to acquire from animals and it is invasive. There is much debate on the ethics of conducting genetic research on stored tissue samples (Clayton et al., 1995, Cambon-Thomsen, 2004, McGuire and Beskow, 2010). There are a wide variety of DNA sources that are non-invasive; the most widely used is dung. Genetic analysis has been successful using dung in a number of species including baboon (*Papio spp*), mountain lion (*Puma concolor*), Eurasian badger (*Meles meles*), grey seal (*Halichoerus grypus*), Abbot's duiker (*Cephalophus spadix*), Harvey's duiker (*Cephalophus harveyi*), Blue duiker (*Philantomba monticola*), bushbuck (*Tragelaphus* spp), wild pig (*Sus scrofa*), and okapi (*Okapia johnstoni*) (Bayes et al., 2000, Ernest et al., 2000, Frantz et al., 2003, Parsons et al., 2005, Bowkett et al., 2009, Kierepka et al., 2016, Stanton et al., 2016).

Vidya and Sukumar (2005a) reported the development of a successful and reliable method for the detection of microsatellites from Asian elephant dung. A study from the same year investigating microsatellites in the African elephant also reports the use of dung successfully (Okello et al., 2005). Dung samples are a preferred sample type when conducting research in species that are sensitive and at risk of extinction. Using dung enables collection of samples with minimal disruption to the animals; this is especially useful when studying animals in the wild (Okello et al., 2005).

6.1.3 Use of genetic testing in elephants

A number of studies have used genetic testing for phylogenetic analysis, such as determining different sub-species of elephants and different populations within the species. This has led to an increased understanding of differing populations and the possibility of including more subspecies of both African and Asian elephants (Fernando et al., 2000, Fleischer et al., 2001, Roca et al., 2001, Eggert et al., 2002, Rohland et al., 2010).

Genetic testing has suggested that there are two species of African elephant; assumptions had previously been based on

morphological evidence. The study examined 195 elephants from 21 populations looking at nuclear genes which equated to 1732 base pairs, and this resulted in two distinct groupings, the savannah elephant (*Loxodonta Africana*) and the forest elephant (*Loxodonta cyclotis*) (Roca et al., 2001). Genetic testing can be useful in providing information used in conservation attempts, mitochondrial DNA obtained from Asian elephants in Borneo has been compared to Asian elephants across other range countries (Fernando et al., 2003). The study determined that Asian elephants in Borneo are genetically distinct from those in other range countries and it was concluded that they have not been introduced there from another location. This highlights the use of genetic testing in the confirmation of genetic distinctiveness in certain species (Fernando et al., 2003).

6.1.4 Determining parentage of elephants

Determining parentage of elephants in captivity can be easier than those in the wild, as a stud book is available for both Asian and African elephants; breeding information is usually routinely recorded by zoos. A number of studies have used microsatellite markers for determining parentage of both African and Asian elephants; this is useful in wild animals as parentage is usually not known. A number of microsatellite loci

have been identified in the African elephant, whereas a limited number have been determined in their Asian counterpart. There are a number of examples of loci first being used in the African elephant that have then shown to also be useful in the Asian elephant (Nyakaana and Arctander, 1998, Comstock et al., 2000, Eggert et al., 2000, Comstock et al., 2002). Microsatellites have been used to determine the genetics of various populations of elephants; this type of study has typically been based on 5 or 6 microsatellites (Whitehouse and Harley, 2001, Fernando et al., 2003, Vidya et al., 2004, Okello et al., 2008). However, to better estimate paternity and kinship, additional microsatellites are required.

Understanding more about captive elephant genetics could be an important factor when looking at contributors to disease. As little is understood about the transmission of EEHV it is also important to further investigate other factors that may contribute to infection such as genetic susceptibility and disease resistance.

6.2 Materials and methods

6.2.1 Animals in this study

A total of 16 Asian elephants were used in this study. Tissue samples were obtained from five dead Asian elephants located

in the UK; three of the deaths were thought to be associated with EEHV. Trunk wash samples were collected from four living animals located in the UK. Faeces was also collected from seven living animals located in Oklahoma, USA (Table 16).

Table 16: Details of animals used in this study, animal status and age was determined at the start of the study (August 2015).

Animal ID	Status	Cause of death	Age (Years)	Location	Sex	Sample type
UON_1329	Dead	EEHV	3	UK	Male	Tissue
UON_1189	Dead	EEHV	2	UK	Female	Tissue
UON_1149	Dead	EEHV	2	UK	Male	Tissue
UON_1490	Dead	Unknown	0	UK	Female	Tissue
UON_1002	Dead	Unknown	0	UK	Female	Tissue
UON_1353	Alive	N/A	20	UK	Female	Trunk wash
UON_1309	Alive	N/A	30	UK	Female	Trunk wash
UON_1579	Alive	N/A	31	UK	Female	Trunk wash
UON_1543	Alive	N/A	17	UK	Female	Trunk wash
UON_1026	Alive	N/A	20	USA	Female	Faeces
UON_1094	Alive	N/A	18	USA	Female	Faeces
UON_1285	Alive	N/A	4	USA	Female	Faeces
UON_1622	Alive	N/A	4 months	USA	Female	Faeces
UON_1405	Alive	N/A	46	USA	Male	Faeces
UON_1621	Alive	N/A	48	USA	Female	Faeces
UON_1091	Alive	N/A	36	USA	Female	Faeces

6.2.2 Genogram

6.2.3 Data collection

Elephant data was collected until March 2013 from http://www.asianelephant.net/database.htm and www.elephant.se. This included name, sex, current location,

and date of death if applicable, if the death was caused by EEHV, name of mother and name of father for 621 elephants.

6.2.4 Analysis

Genograms were created using specialised software Cranefoot V3.2 (http://www.finndiane.fi/software/cranefoot/); standard settings were used, and inbreeding was analysed using FSPEED v2.04a (http://fspeed.software.informer.com/) standard settings were also used for this software. The epidemiology of EEHV related deaths was analysed using GraphPad.

6.2.5 Inbreeding

The inbreeding coefficient (F) is the probability of autozygosity, where both allele copies are identical by descent. In this study the F value is expressed as a decimal, it may also be expressed as a percentage. If an animal has an F value of 0.25 it has been mated to its own parent or is a full sibling mating and is 25% homozygous, 0.125 (12.5%) suggests a half sibling mating, grandparent/grandchild mating, uncle/niece or aunt/nephew mating and 0.0625 (6.25%) which results from a great-grandparent/great-grandchild mating, half-uncle/niece, half-aunt/nephew or first cousin mating, an F value of 0.031 (3.1%) suggests the parents have a common great-grand parent.

6.2.6 Microsatellites

6.2.6.1 Sample collection

6.2.6.1.1 Tissue

Tissue samples were collected as described in section 2.1.2

6.2.6.1.2 Trunk washes

Trunk wash samples were collected from nine Asian elephants as described in section 2.1.1

6.2.6.1.3 Faeces

To determine if a non-invasive method is a useful tool, faeces was collected from seven animals; faeces were collected from the centre of the bolus using a swab, and then smeared onto FTA Whatmans cards. Cards were then stored at ambient temperature until processing. Faecal samples were collected from seven Asian elephants located at Oklahoma City Zoo, USA on 23/08/2015.

6.2.6.2 DNA extraction

6.2.6.2.1 Tissue

DNA was extracted from tissue samples as described in section 2.2.1

6.2.6.2.2 Trunk washes

DNA was extracted from trunk wash samples as described in section 2.2.1

6.2.6.2.3 Faeces

Elephant DNA was isolated from faecal samples using QIAamp DNA stool mini kit (Qiagen, Germany), following an amended version of the manufacturer's instructions for the isolation of DNA from stool for pathogen detection. The following amendments were made for a second attempt due no sample remaining when adding one inhibitEX tablet: A 3mm punch a mat (Harris puncher) was used to remove ten punches from the FTA card and these were transferred to a 2ml centrifuge tube. The punches were soaked in PBS overnight. To remove as much faeces from the card as possible, a steel bead beater was added along with buffer ASL, and then incubated at 70°C in a heat block; samples were vortexed, at 30 minute intervals. The amount of inhibited tablet suggested by the manufacturer's instructions (1x tablet per tube) was reduced by half. All samples were centrifuged at 11,000 rpm 10 minutes prior to after the addition of half an inhibitEX tablet. Half the amount of elution buffer AE was used; the elution buffer was prewarmed to 70°C before use, to ensure maximum DNA yield.

6.2.6.3 Trunk wash DNA concentration

A pooled sample of DNA obtained from trunk washes was made for each animal; 5 or 6 samples per animal were pooled. Samples were concentrated using vacuum concentration and DNA precipitation. For the vacuum concentration method, the pooled samples were placed into a vacuum concentrator (Barnstead Genevac mi vac concentrator) for 40 mins at 60°C; this procedure reduced the volume of the sample by half. For the DNA precipitation method, the pooled DNA was transferred into a 1.5ml microcentrifuge tube, where it should occupy less than one quarter of the total volume (250µl was used). To equalize the ion concentration, one tenth of the volume of DNA of 3µM sodium acetate buffer was added to the tube. Two to three volumes of cold 100% ethanol was then added, the samples were then vortexed to mix and placed in a -20°C freezer overnight. The following morning, the samples were removed from the freezer, thawed at ambient temperature and centrifuged at 13,000rpm. The supernatant was then removed. To wash the DNA pellet, 250µl of 4°C 70% ethanol was added and the contents centrifuged for 5 minutes. The supernatant was then removed, and the remaining ethanol is evaporated using a 37°C water bath. The pellet was then resuspended in 50ul sterile water.

6.2.6.4 DNA and inhibitor check PCR

PCR was used to determine if the DNA in the samples was degraded and to see if there were any inhibitors present that would affect the ability of the PCR to amplify DNA in both pooled Asian elephant samples and pooled African elephant samples. The primers (Forward 5”-3” = GCGTGAAGACCCTTGAGGAA and reverse 5”-3” = TGTTCATCTGTCGCTTCCTG (Eurofins, Germany)) used were expected to amplify IFNγ and produce a PCR product of 100bp in size. Each reaction consisted of 1µl of each primer, 0.8µl dNTP mix, 0.6µl 50mM $MgCl_2$, 1µl standard 10x buffer (New England Biolabs, USA), 0.1µl taq (New England Biolabs, USA) and 4.4µl sterile water. Cycling conditions were as follows: 94^{o}C for 1 min followed by 25 cycles of 94^{o}C for 30 sec, 60^{o}C for 30s and 72^{o}C for 1 min, followed by a final extension of 3 min at 72^{o}C.

6.2.6.5 Amplification of microsatellites using PCR

Fourteen microsatellite loci were amplified from elephant DNA using amended protocols from previous publications. The amendments included labelling forward primers with a fluorescent dye, thus replacing the use of radioactively labelled primers. This was done to allow the analysis of PCR products by length. Primers are detailed in Table 17. Each well of a 96 well plate contained three PCR products labelled with

either 6-FAM, ROX or HEX; the ladder (LIZ 500) was also included. PCR products were sent to Source Bioscience for fragment length analysis.

Table 17: Microsatellite loci used to determine genetic diversity.

Well number	Loci	Expected size (bp)	Florescent dye	Forward primer	Reverse primer	Species	Reference
1	EMX-1	152	6-FAM	AGGACTTATTTGCTTAGATGG	AGGCAATGTTTCGTTCTGT	*E maximus*	Fernando et al. (2001)
1	EMX-3	254	HEX	CATGGTTAACTCATTGCTTGC	GTGTTCCCTCCCTCTCATCAT	*E maximus*	Fernando et al. (2001)
1	LA2	227	ROX	CTTGGTGGGAGTCATGACCT	GGAGAAATGACTGCCCGATA	*E maximus and L africana*	Eggert et al. (2002)
2	LA4	130	6-FAM	GCTACAGAGGACATTACCCAGC	TTTCCTCAGGGATTGGGAG	*E maximus and L africana*	Eggert et al. (2002)
2	FH48	178	HEX	GAGTCTCCATAATCAAGAGCG	CCTCCCTGGAATCTGTACAG	*L africana*	Comstock et al. (2000)
2	FH60	148	ROX	CAAGAAGCTTTGGGATTGGG	CCTGCAGCTCAGAACACCTG	*L africana*	Comstock et al. (2000)
3	FH94	229	6-FAM	TTCCTCCCACAGAGCAGC	ATTGGTTAATTTGCCAGTCCC	*L africana*	Comstock et al. (2000)
3	FH103	154	HEX	TGTGCTGCCACTTCCTACAC	GATGTTGAGACAGTTCTGTAAG	*L africana*	Comstock et al. (2000)
N/A	FH102		ROX	CTTCATTACTGACCTAAACGAG	GGACAGGGCTGGAGAAATATG	*L africana*	Comstock et al. (2000)
N/A	FH127		6-FAM	ACTGACCGGGAAGAGGAAGT	AGGTTTCTAAGCTGAATTGG	*L africana*	Comstock et al. (2002)
4	FH153	169	HEX	CATGGGCCTAAGCTAAAACG	GTCACATGGGGTTGCTAC	*L africana*	Comstock et al. (2002)
4	LafMS02	147	ROX	GAAACCACAACTTGAAGGG	TCGCTTGTAAGAAGGCGTG	*L africana*	Nyakaana and Arctander (1998)
5	LafMS03	144	6-FAM	CATATGAACATACCGGAAC	GAAACTCCTCGAGTAGTAGAA	*L africana*	Nyakaana and Arctander (1998)
5	LafMS05	158	HEX	CCTTAGGCTGGGTTGTAT	AATGGACTTGGGACTTGCCAAAATGT	*L africana*	Nyakaana and Arctander (1998)

6.2.6.6 Optimised reaction and cycling conditions

Reaction and cycling conditions were amended and optimised from published protocols; all loci utilised the same cycling conditions (Table 18). The annealing temp was optimised for each locus (Table 18). All reaction volumes were 10µl, consisting of; 1µl 10x buffer (New England biolabs, USA), 0.8µl dNTP mix (New England biolabs, USA), and 0.1µl taq (New England biolabs, USA) and a variable amount of $MgCl_2$ (New England biolabs, USA) (Table 18).

Table 18: Optimised cycling conditions for all reactions.

Step	Temperature (^oC)	Time
Initial denaturation	94	4 min
Denaturation	94	30 sec
Annealing	Variable	30 sec
Extension	72	1 min
Final extension	72	3 min
Final hold	4	Indefinitely

PCR reactions for LA2 and LA4 were conduction in an Applied Biosystems 2720 thermal cycler, and the remaining PCRs were conducted in a Techne TC-512 thermal cycler Machine. All PCR products were visualised using gel electrophoresis on a 2% agarose gel using the fluorescent dye Nancy 520.

6.2.6.6.1 Reaction conditions

A number of modifications were made to the primer concentration if the amount stated in the original publication did not appear to produce a PCR product, this was then determined using a concentration gradient. $MgCl_2$ concentration and annealing temperatures were also optimised; the final optimised primer concentrations and annealing temperature used in this study can be seen in Table 19.

Table 19: Concentrations and annealing temperatures used in PCR reactions

Loci	Primer concentration	$MgCL_2$ Concentration	Annealing temperature
EMX-1	0.25µM	1.5mM	62.2
EMX-3	0.25µM	1.5mM	66.1
LA2	0.2µM	2mM	58
LA4	0.2µM	4mM	54
FH48	10µM	2mM	58
FH60	0.2µM	1.5mM	59
FH94	10µM	1.5mM	61
FH103	10µM	1.5mM	66.6
FH153	10µM	1.5mM	58
LafMS02	0.2µM	1.5mM	54
LafMS03	0.2µM	4mM	54
LafMS04	0.2µM	1.5mM	54

6.2.6.7 Analysis of fragment length analysis

All traces were analysed using GeneMarker (Version 2.6.7) software.

6.3 Results

6.3.1 Genogram

Data from a total of 621 Asian elephants were included in this study, including 246 males and 357 females; the sex was unknown for 18 Asian elephants. Four animals were of unknown location, this was due to the limitations of the software, where one parent was known it required both to be included, and the sex of the unknown parent was known.

A total of 136 males were located in Europe and 105 in the North America, 202 females were located in Europe, 153 in North America and four males of unknown location. Sex was unknown for a total of 18 elephants, with 13 located in Europe and 5 in the North America. A total of 39 deaths were recorded as being associated with EEHV, 20 in Europe and 19 in the North America.

Preliminary data suggests the possibility of a familial link in EEHV cases. Certain clusters within the genogram showed a number of deaths occurring within the cluster. In a cluster of ten Asian elephants of which three were alive and seven were dead, five of the deaths were thought to be associated with EEHV (Figure 21). However, as the elephants were originally

located at the same zoo, location association also needs to be considered.

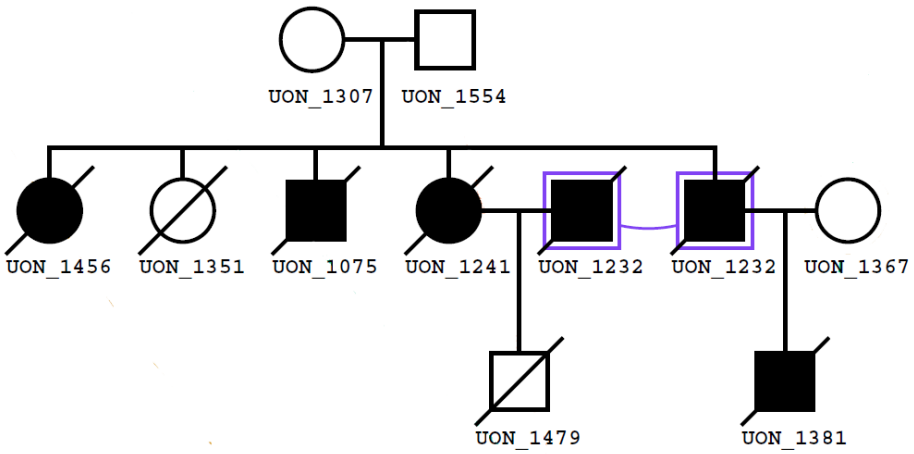


Figure 60: A cluster showing a large number of EEHV associated deaths. This cluster shows ten related Asian elephants, three alive and seven dead. Of the seven that were dead, five were thought to be deaths associated with EEHV. Circle = female, and square = male, a line through shape = dead, and black shape = death associated with EEHV.

Other clusters of related elephants within the genogram showed no deaths associated with EEHV (Figure 22).

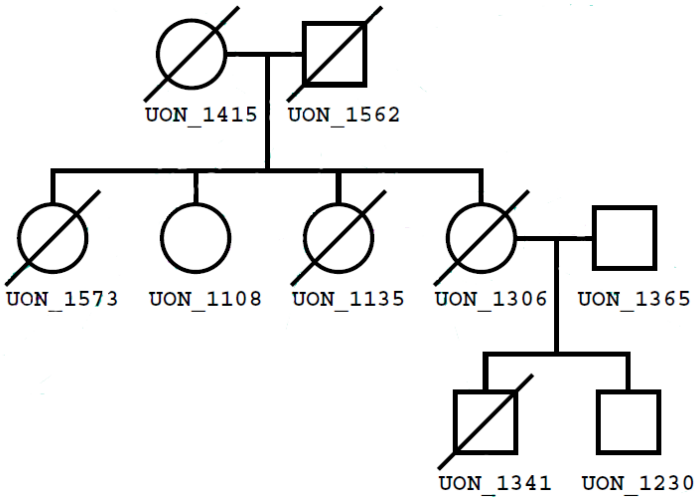


Figure 61: A cluster showing no EEHV associated deaths. This cluster shows nine related Asian elephants, three of which were currently living and six that were dead, of which none of the deaths were reported to be associated with EEHV. Circle = female, and square = male, and a line through shape = dead.

6.3.2 Inbreeding

Inbreeding coefficients (F) were calculated for 445 animals with known parents, this value is the percentage of alleles that are homozygous. Of the 445 elephants, 15 had an F value of >25% and 19 had a value of >12.5%; the mean F value for all elephants was 0.9%. Of the European elephants, eight had an F value of >25% and 11 had an F value of >12.5%; and the mean for all European animals was 0.9%. A smaller number of animals in North America had F values above 12.5%, with seven animals with F value >25% and eight >12.5%; the mean F value for all North America elephants was 0.9% (Table 20).

Table 20: Inbreeding coefficients (F) for Asian elephants with known parents.

Total	Inbreeding mean	0.0094 (0.94%)
	>25%	15
	>12.5%	19
European	Inbreeding mean	0.0093 (0.93%)
	>25%	8
	12.5 - 24%	11
North America	Inbreeding mean	0.0097 (0.97%)
	>25%	7
	12.5 - 24%	8

6.3.3 Analysis of deaths

A total of 39 deaths were reported to be associated with EEHV; slightly more male deaths were associated with EEHV (22) in comparison to females (17). The highest number of

recorded deaths occurred in the USA, with 15 being reported (38.46%), this is followed by ten (25.64%) in the UK, five (12.82%) in Germany, three (7.69%) each in Canada and Switzerland, and one (2.56%) each in Denmark, the Netherlands and France (Figure 23).

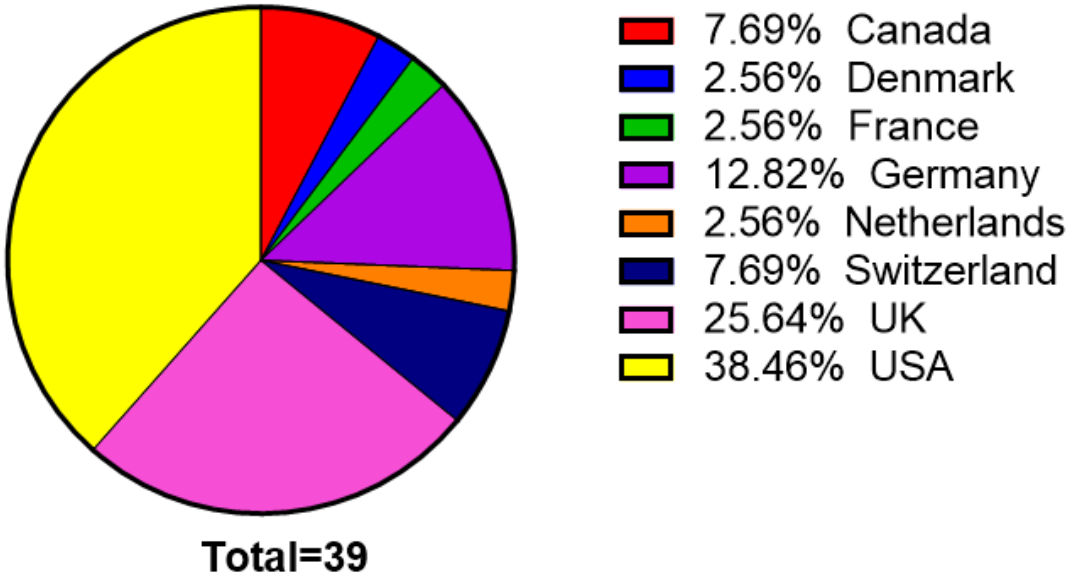


Figure 62: Percentage of EEHV related deaths by country.

The mean age of death was 3.1 years of age, whilst the mode was 2 years old. In females, the mean age of death was 3.6, while in males, it was 2.7 years old (Table 21).

Table 21: Mean, median and mode of age at death for deaths reported to be associated with EEHV

	Mean	Median	Mode
All animals	3.2	2	2
Males	2.7	2	2
Females	3.6	2	2

6.3.4 Microsatellites

6.3.4.1.1 DNA concentration for trunk washes

6.3.4.1.1.1 Vacuum concentrator method

A vacuum concentrator uses evaporation to concentrate the DNA sample. Concentrations of most samples increased after concentration; however, one sample showed an apparent decrease from 2.903ng/µl to 1.500ng/µl (Table 22). The concentrations measured may not reflect the actual concentration, as the Nanodrop machine can be inaccurate or affected by sample contamination.

Table 22: DNA concentrations of pooled Asian elephant DNA before and after vacuum concentration.

Animal ID	Before (ng/µl)	After (ng/µl)
UON_1140	15.28	35.02
UON_1093	2.903	1.500
UON_1625	Undetectable	76.43
UON_1089	Undetectable	82.85
UON_1175	0.3316	39.07
UON_1543	50.04	206.0
UON_1579	6.181	73.51
UON_1309	8.899	53.31
UON_1353	12.71	86.19

6.3.4.1.1.2 Precipitation method

Similarly, the precipitation method in most cases increased the amount of DNA present within the sample. All but one sample saw an increase in the amount of DNA present; however, sample UON_1579's quantity decreased from 7.213ug/µl to 1.100ug/µl (Table 23).

Table 23: DNA concentrations before and after concentration by DNA precipitation.

Animal ID	Before (ng/µl)	After
UON_1140	4.692	21.62
UON_1093	3.893	14.08
UON_1625	7.680	49.67
UON_1089	4.207	17.44
UON_1175	5.799	22.45
UON_1543	34.85	208.8
UON_1579	7.213	1.100
UON_1309	9.885	55.72
UON_1353	16.12	61.02

6.3.4.1.2 DNA quality and inhibitors in pooled tissue samples

A pooled sample (pooled samples were either pooled DNA from tissue samples or from trunk washes) was made for each group of elephants, one Asian group (nine animals) and one African group (five animals); the presence of DNA in the pooled samples and the lack of inhibitors was confirmed using

PCR to amplify IFNγ. A product of the expected size (100bp) was observed in each sample (Figure 24).

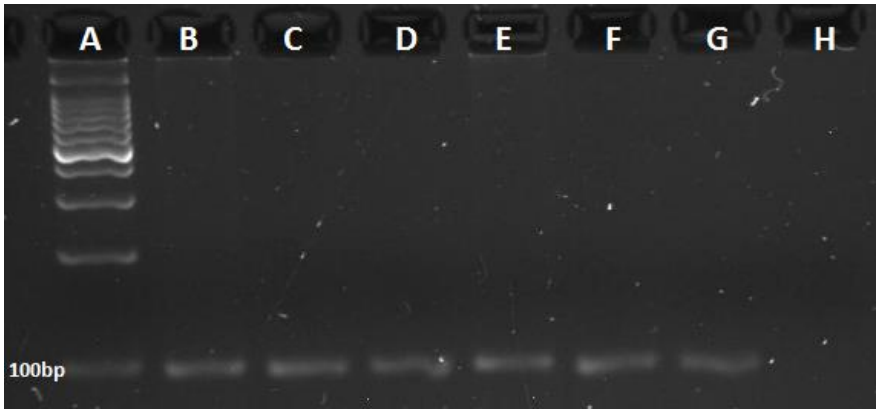


Figure 63: Visualisation of a PCR on a 2% agarose gel to confirm the presence of Asian elephant and African elephant DNA. Lane A: 100bp ladder, B: Asian elephant pooled sample pure, C: Asian elephant pooled sample 1/10 dilution, D: Asian elephant pooled sample 1/100 dilution, E: African elephant pure, F: African elephant pooled sample 1/10, G: African elephant pooled sample 1/100 dilution and H: negative control.

6.3.4.2 Optimisation of microsatellite PCR's results by locus

6.3.4.2.1 LA2 PCR

To initially optimise the LA2 locus, the primer concentration was lowered from 10mM to 0.2mM; however, this did not result in a product when visualised on an agarose gel (not shown). To determine the optimal $MgCl^2$ concentration for this locus, an $MgCl_2$ dilution series (1.5mM, 2mM, 3mM and 4mM) was performed using pooled Asian and African elephant samples. A concentration of 2mM for the Asian elephant samples, and 4mM for the African samples produced a PCR product of the correct size (Figure 25).

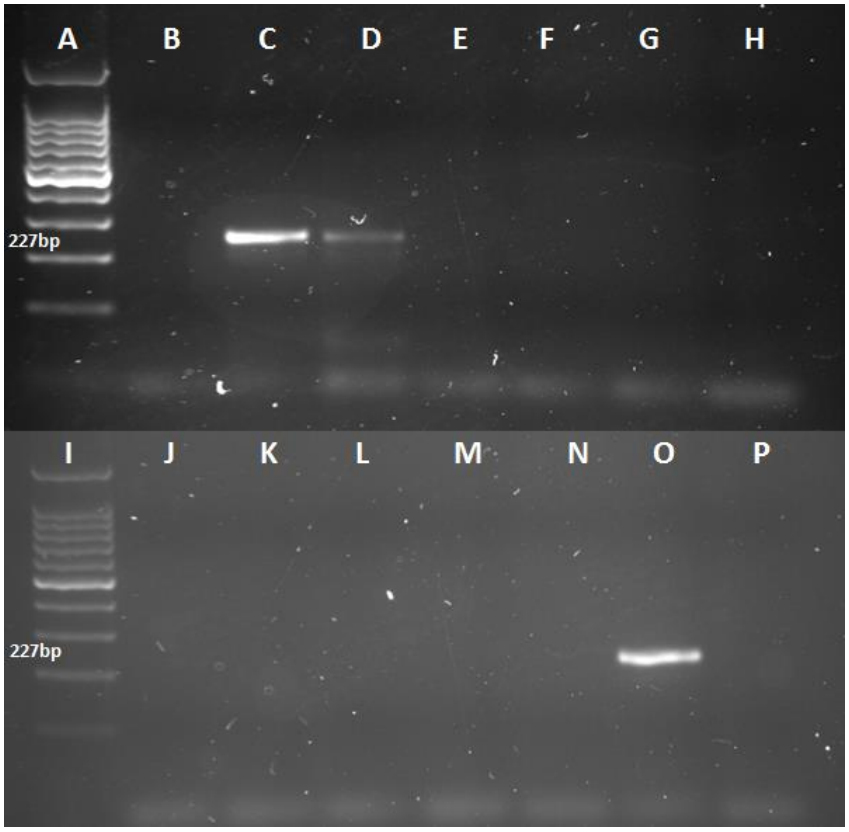


Figure 64: Visualisation of a PCR on a 2% agarose gel to determine if changing the concentration of $MgCL_2$ optimised the reaction for locus LA2. Top image: Visualisation of PCR reaction using primers to amplify the LA2 microsatellite locus using a 1/10 dilution of pooled Asian elephant sample. Lane A: 100bp ladder (Promega), lanes B-G are a series of $MgCL_2$ concentrations; B: 1.5mM, C: 2mM, D: 2.5mM, E: 3mM, F: 3.5mM, G: 4mM and H: negative control. A PCR product of the expected band size of 227bp can be seen in lanes C and D, lane D also shows some smearing. Bottom image: The reaction is as described above but using a 1/10 dilution of the African elephant pooled sample. Lane I: 100bp ladder (promega), lanes J-O concentrations as described above and lane P: negative control.

6.3.4.2.2 LA4 PCR

Optimisation included performing an $MgCl_2$ dilution series to determine the optimal conditions for this PCR reaction. The PCR was performed using pooled Asian elephant DNA and pooled African elephant DNA. A PCR product of the correct size was not visualised for the Asian elephant sample, however, there was a product of the correct size (130bp) which observed for the African elephants pooled sample; this was at

a high concentration (4mM) of $MgCl_2$. The agar gel is heavily marked in comparison to the other gels in this study, this may be due to the brightness settings on the gel viewer or there may have been contamination when preparing the gel (Figure 26).

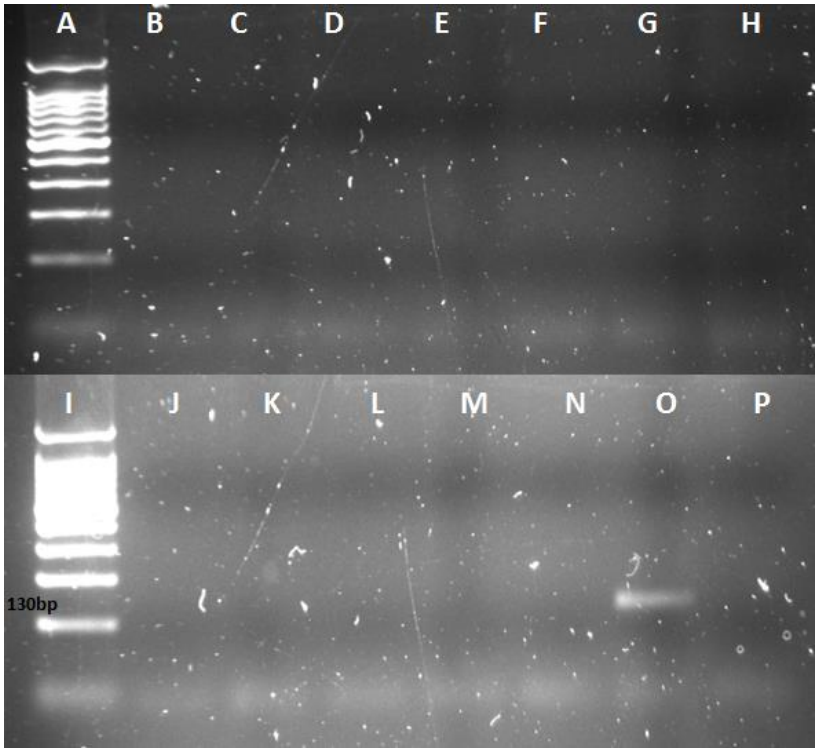


Figure 65: Visualisation of a PCR on a 2% agarose gel to determine if changing the concentration of $MgCL_2$ optimised the reaction for locus LA4. Top image: Visualisation of PCR reaction using primers to amplify the LA4 microsatellite locus using a 1/10 dilution of pooled Asian elephant sample. Lane A: 100bp ladder (Promega), lanes B-G are a series of $MgCL_2$ concentrations; B: 1.5mM, C: 2mM, D: 2.5mM, E: 3mM, F: 3.5mM, G: 4mM and H: negative control. Bottom image: The reaction is as described above but using a 1/10 dilution of the African elephant pooled sample. Lane I: 100bp ladder (Promega), lanes J-O concentrations as described above and lane P: negative control. A PCR product is visible on the bottom image at an expected bp size of 130bp and at a concentration of 4mM.

This led to further investigation which then included replacing the pooled Asian and African elephant samples with DNA samples from individual animals, an $MgCl_2$ of 4mM was used. PCR products of the correct size were visualised for each

animal, suggesting that this locus is present in each animal. However, nonspecific products were evident in most lanes, and there was also evidence of smearing which could be due to high concentrations of DNA present (Figure 27).

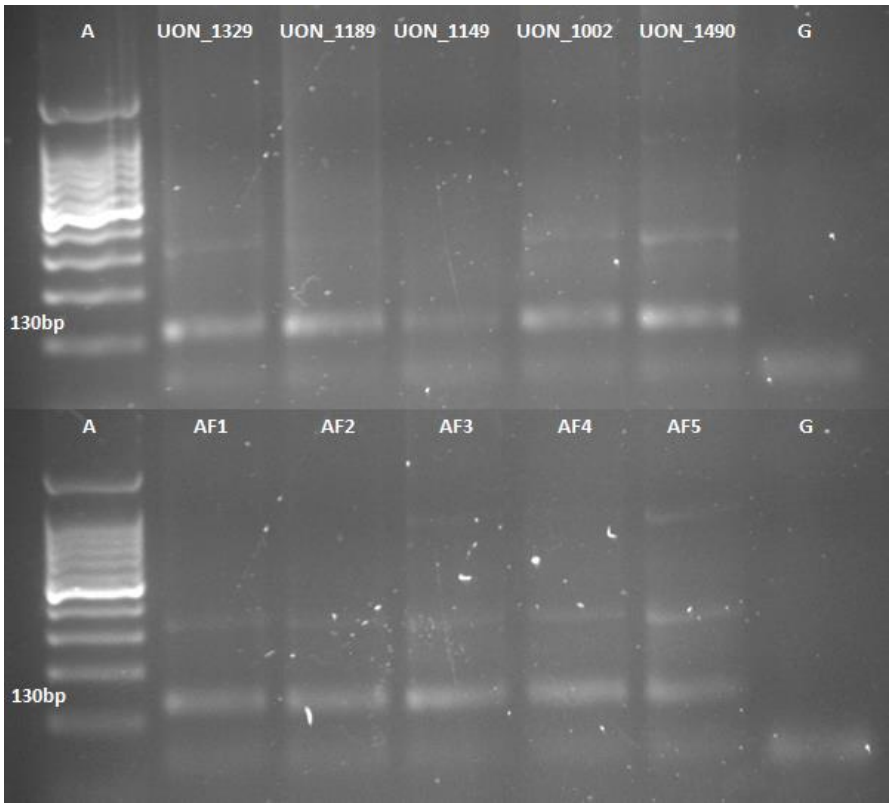


Figure 66: Visualisation of a PCR on a 2% agarose gel to determine if locus LA4 is present in individual animals at an $MgCl_2$ concentration of 4mM. Top image: using a 1/10 dilution of DNA from each individual Asian elephant in a reaction using primers to amplify locus LA4. Lane A: 100bp ladder (promega) and Lane G: negative control. A PCR product of the expected size of 130bp is visible for Asian elephant. There are also some other bands present in lanes UON_1329, UON_1002 and UON_1490. Bottom image: using a 1/10 dilution of DNA from each individual African elephant, lane A: a100bp ladder (Promega) G: negative control. A PCR product of the expected size (130bp) was also observed for each animal.

6.3.4.2.3 EMX-1 and subsequent loci PCR

To optimise this PCR, a temperature gradient and an $MgCl_2$ series was combined into the same experiment. For the locus EMX-1, a primer concentration of 10µm was used as detailed in Fernando et al. (2001); a temperature gradient of 61.9-

66.1°C was performed at three different $MgCl_2$ concentrations: 1.5mM, 2mM and 4mM. The PCR was visualised using a 2% gel; a PCR product of the correct size was present in lane C: the reaction contained 1.5mM of $MgCl_2$ and was run at a temperature of 62.2°C. Smearing and non-specific binding were present in lane D, this temperature was not used in the final reaction, this consisted of a temperature of 62.6°C and an $MgCl_2$ concentration of 1.5mM (Figure 28).

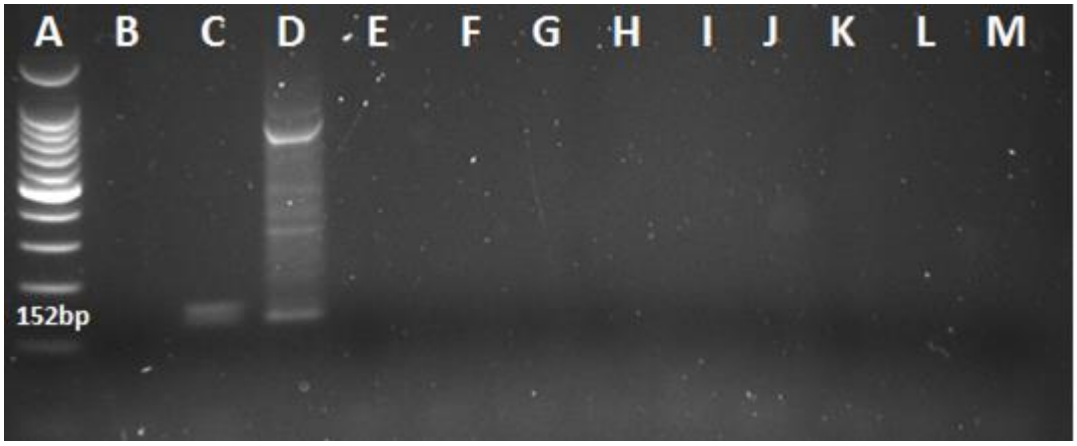


Figure 67: Visualisation of a PCR on a 2% agarose gel to determine the optimal reaction conditions for locus EMX-1 using pooled Asian elephant DNA. Lane A is 100bp ladder (Promega), and lanes B-M show a temperature gradient in degrees C of B: 61.9, C: 62.2, D: 62.6, E: 63, F: 63.4, G: 63.8, H: 64.1, I: 64.8, J: 65.1, K: 65.6, L: 66.1 and M: 66.1. The reactions contained 1.5mM of $MgCL_2$, and a PCR product of the expected size (152bp) was visualised in column C.

6.3.4.2.4 EMX-3 PCR

Optimisation for locus EMX-3 included trialling three different $MgCl_2$ concentrations (1.5mM, 2mM and 4mM) and a temperature gradient (61.9-66.1°C). The final optimised reaction used 1.5mM $MgCl_2$ and a temperature of 64°C. However, all reactions across the different temperatures with

an $MgCl_2$ concentration of 1.5mM produced a correct product size of 254bp (Figure 29). Bands of the correct size were also observed in all lanes when using an $MgCl_2$ concentration of 2mM and in nine lanes when using a concentration of 4mM; however, as the $MgCl_2$ concentration increased so did the amount of visible primer dimer (not shown).

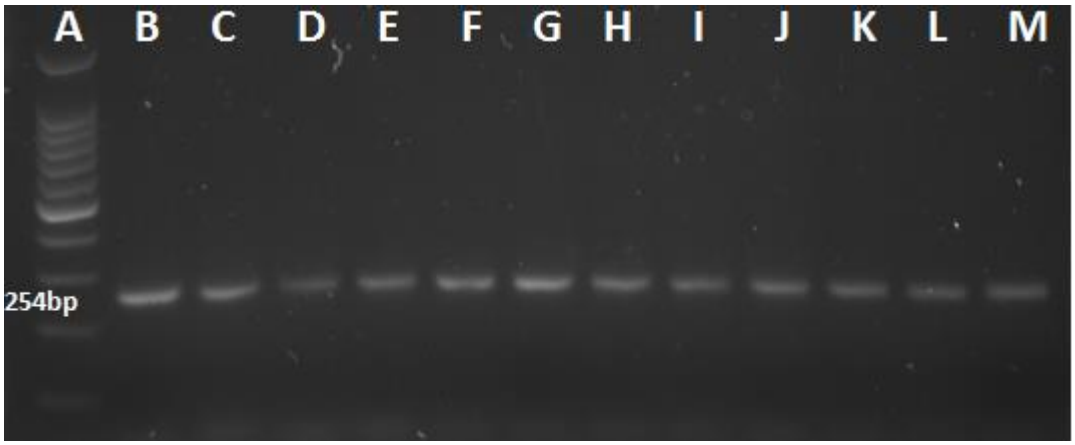


Figure 68: Visualisation of a PCR on a 2% agarose gel to determine the optimal reaction conditions for locus EMX-3 using pooled Asian elephant DNA. Lane A is 100bp ladder (Promega), and lanes B-M show a temperature gradient in degrees C of B: 61.9, C: 62.2, D: 62.6, E: 63, F: 63.4, G: 63.8, H: 64.1, I: 64.8, J: 65.1, K: 65.6, L: 66.1 and M: 66.1. The reactions contained 1.5mM of $MgCL_2$, a PCR product of the expected size (254bp) was visualised in all lanes.

6.3.4.2.5 FH48 PCR

A PCR using a temperature gradient (55.9-60.1^{o}C) and differing $MgCl_2$ concentrations (1.5mM, 2mM and 4mM) was performed to optimise the reaction for locus FH48. Using an $MgCl_2$ concentration of 2mM resulted in products of the correct size (178bp) (Figure 30). A concentration of 58°C was selected for the final optimised reaction along with a primer concentration of 10µM and an $MgCl_2$ of 2mM. Bands of the

correct size were visible in all lanes at an $MgCl_2$ concentration of 2mM; at a concentration of 4mM, the reaction did not produce any PCR products when incubated above 65°C, and primer dimer was visible at lower temperatures (not shown)

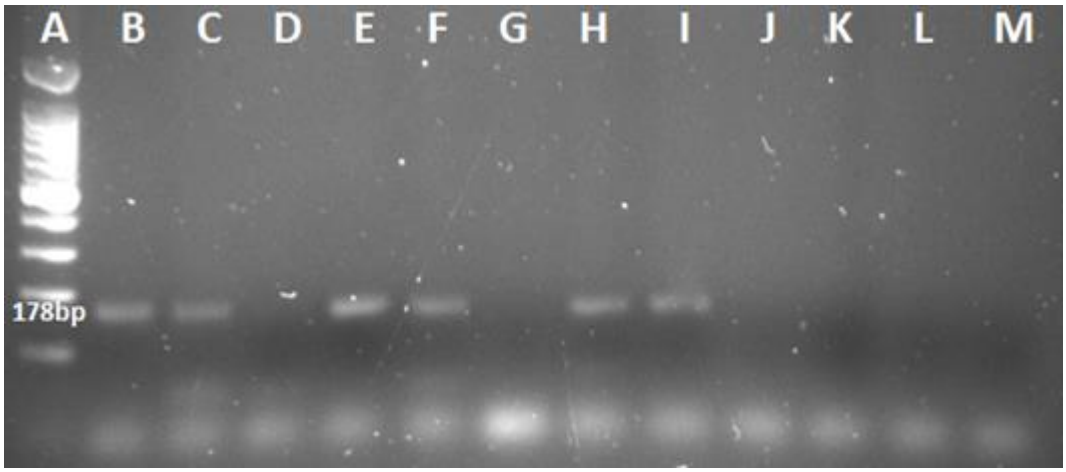


Figure 69: Visualisation of a PCR on a 2% agarose gel to determine the optimal reaction conditions for locus FH48 using pooled Asian elephant DNA. Lane A is 100bp ladder (Promega), and lanes B-M show a temperature gradient in degrees C of B: 55.9, C: 56.2, D: 56.6, E: 57, F: 57.4, G: 57.8, H: 58.1, I: 58.8, J: 59.1, K: 59.6, L: 60.1 and M: 60.1. The reactions contained 2mM of $MgCL_2$, a PCR product of the correct size (178bp) was visualised in lanes B, C, E, F, H and I.

6.3.4.2.6 FH60 PCR

PCR optimisation for locus FH60 was unsuccessful; the first attempt included both a temperature gradient from 54-64°C and three different $MgCl_2$ concentrations of 1.5mM, 2mM and 4mM. PCR products were not visualised at any of the temperatures within the gradient and at any $MgCL_2$ concentrations used (not shown).

Further optimisation included reducing the concentration of both forward and reverse primers from 10µm to 0.2µm this

resulted in a number of non-specific bands at an $MgCL_2$ concentration of 4mM; no PCR products of the correct size were visualised at concentrations of 1.5mM or 2mM (Figure 31).

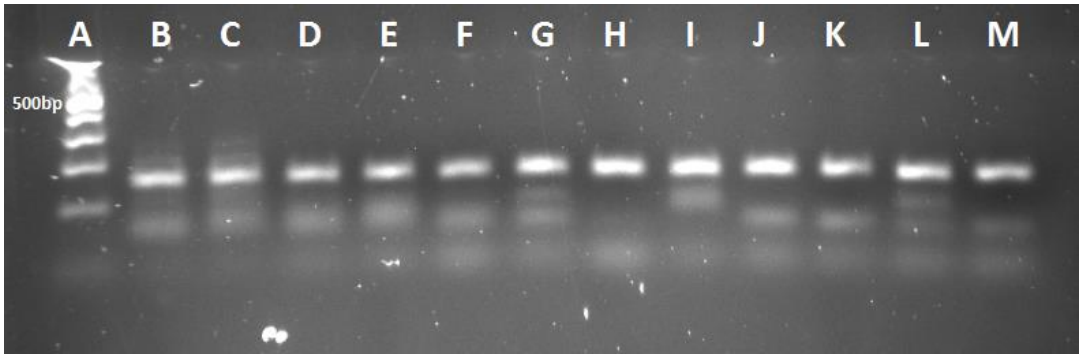


Figure 70: Visualisation of a PCR on a 2% agarose gel to determine the optimal reaction conditions for locus FH60 using pooled Asian elephant DNA. Lane A is 100bp ladder (Promega), and lanes B-M show a temperature gradient in °C of B: 54, C: 54.6, D: 55.6, E: 56.7, F: 57.5, G: 58.5, H: 59.3, I: 60.9, J: 61.6, K: 62.9, L: 64.1 and M: 64. The reactions contained 4mM of $MgCL_2$, a number of non-specific PCR products of the incorrect size were visualised in all lanes.

6.3.4.2.7 FH94 PCR

A temperature gradient (58.9-63.1°C) and 3 different $MgCL_2$ (1.5mM, 2mM and 4mM) were used to optimise the PCR reaction for loci FH94. Bands of the correct size (178bp) were visualised in all wells; however, at an $MgCl_2$ concentration of 1.5mM, there was smearing visible in most lanes, with the most occurring in lanes D, E and F (Figure 32). At concentrations 2mM and 4mM, there was an increased amount of smearing, and primer dimer was also visible (not shown). A final primer concentration of 10µl at an $MgCl_2$ concentration of

1.5mM and a temperature of 59°C were selected for use in the final reaction.

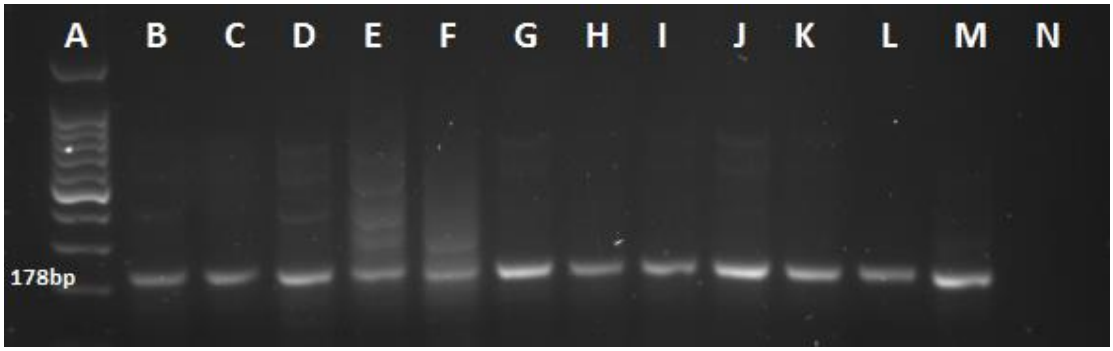


Figure 71: Visualisation of a PCR on a 2% agarose gel to determine the optimal reaction conditions for locus FH94 using pooled Asian elephant DNA. Lane A is 100bp ladder (Promega), and lanes B-M show a temperature gradient in degrees C of B: 58.9, C: 59.2, D: 59.8, E: 60, F: 60.4, G: 60.8, H: 61.1, I: 61.8, J: 62.1, K: 62.6, L: 63.1 and M: 63.1. The reactions contained 1.5mM of $MgCL_2$, a PCR product of the correct size (229bp) was visualised in lanes in all lanes.

6.3.4.2.8 FH103 PCR

Both a temperature gradient (55.9-60.1^{o}C) and 3 different $MgCl_2$ concentrations (1.5mM, 2mM and 4mM) were used to optimise the PCR reaction for loci FH103. A product of the correct size (154bp) was observed in one lane at an $MgCL_2$ concentration of 1.5mM (Figure 33). No bands were visible at concentrations of 2mM or 4mM; however, at 4mM primer dimer was visible (not shown). A primer concentration of 10µl at an $MgCL_2$ concentration of 1.5mM and a temperature of 56.6^{o}C was used in the final reaction.

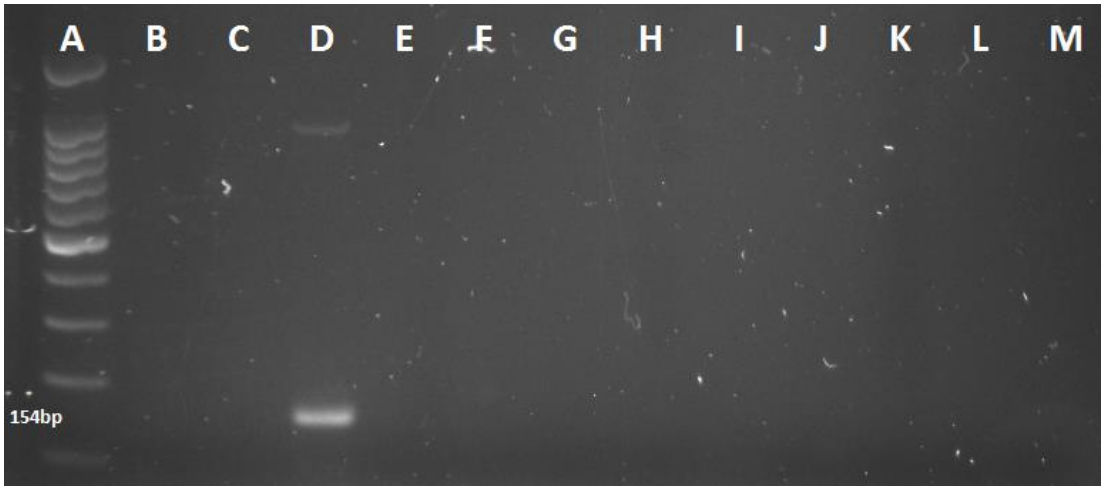


Figure 72: Visualisation of a PCR on a 2% agarose gel to determine the optimal reaction conditions for locus FH103 using pooled Asian elephant DNA. Lane A is 100bp ladder (Promega), and lanes B-M show a temperature gradient in degrees C of B: 55.9, C: 56.2, D: 56.6, E: 57, F: 57.4, G: 57.8, H: 58.1, I: 58.8, J: 59.1, K: 59.6, L: 60.1 and M: 60.1. The reactions contained 1.5mM of $MgCL_2$, a PCR product of the correct size (154bp) was visualised in lane D.

6.3.4.2.9 FH102 PCR

A temperature gradient (57.9-62.1°C) and at an $MgCl_2$ concentration series (1.5mM, 2mM and 4mM) was performed. No products of the expected size (176bp) were seen. PCR products present in lanes C, D and E were around 140bp and larger bands present in lanes D, E and G are around 200bp (Figure 34). Bands were also visualised at $MgCl_2$ concentrations of 1.5mM and 4mM; a band of around 200bp was present at a temperature of 58.6°C at 1.5mM and a band at around 140bp was visualised in all lanes at 4mM (not shown).

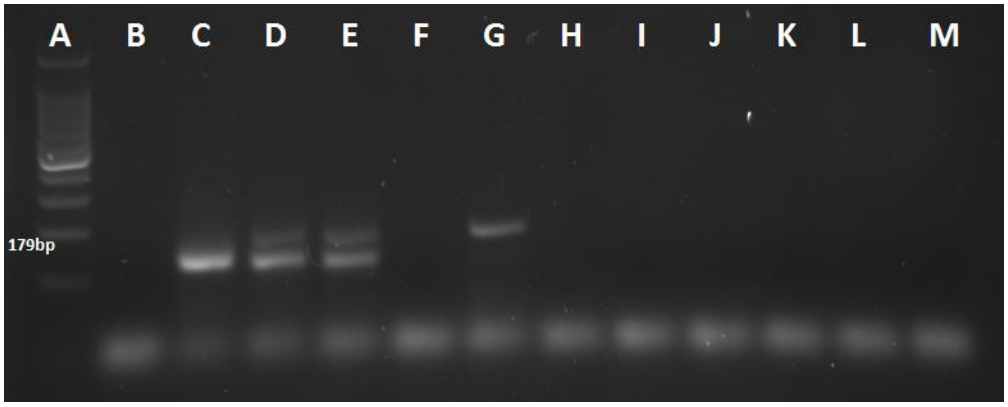


Figure 73: Visualisation of a PCR on a 2% agarose gel to determine the optimal reaction conditions for locus FH102 using pooled Asian elephant DNA. Lane A is 100bp ladder (Promega), and lanes B-M show a temperature gradient in degrees C of B: 57.9, C: 58.2, D: 58.6, E: 59, F: 59.4, G: 59.8, H: 60.1, I: 60.8, J: 61.1, K: 61.6, L: 62.1 and M: 62.1. The reactions contained 2mM of $MgCL_2$, a PCR product of the correct size (179bp) was not visualised.

Further optimisation was carried out for this locus, with the primer concentration being reduced from 10um to 0.2um which was successful in other loci. Non-specific bands of around 200bp and 140bp were visualised at concentrations of 4mM $MgCl_2$ (Figure 35).

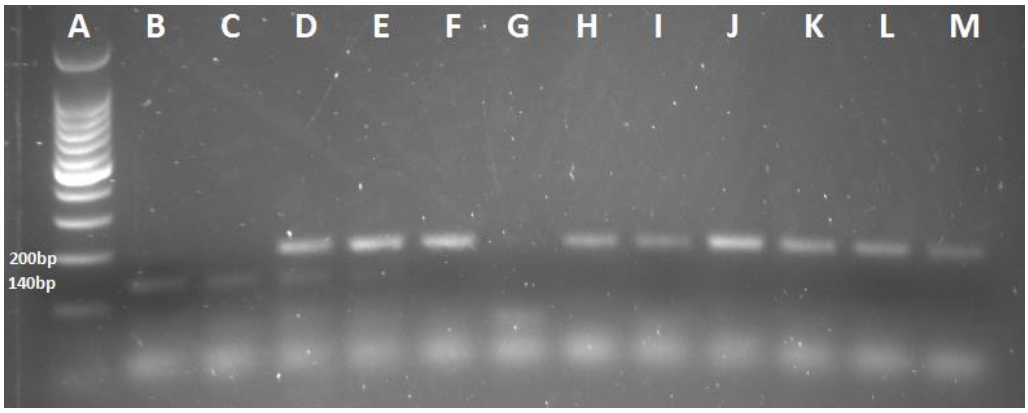


Figure 74: Visualisation of a PCR on a 2% agarose gel to determine if a lower primer concentration produced a PCR product of the correct size for locus FH102. Lane A is 100bp ladder (Promega), and lanes B-M show a temperature gradient in °C of B: 57.9, C: 58.2, D: 58.6, E: 59, F: 59.4, G: 59.8, H: 60.1, I: 60.8, J: 61.1, K: 61.6, L: 62.1 and M: 62.1. The reactions contained 4mM of $MgCL_2$, a PCR product of the correct size (179bp) was not visualised.

6.3.4.2.10 FH127 PCR

A number of optimisations were made to the PCR for this locus; to attempt to determine the optimal conditions, a PCR using a temperature gradient (51-61.1°C) and 3 differing concentrations of $MgCl_2$ (1.5mM, 2mM and 4mM) were used. This resulted in a number of non-specific bands of the incorrect product size in lanes B, C, D, E, F and H at an $MgCl_2$ concentration of 4mM (Figure 36). There were no products visualised at $MgCl_2$ concentrations of 1.5mM and 2mM.

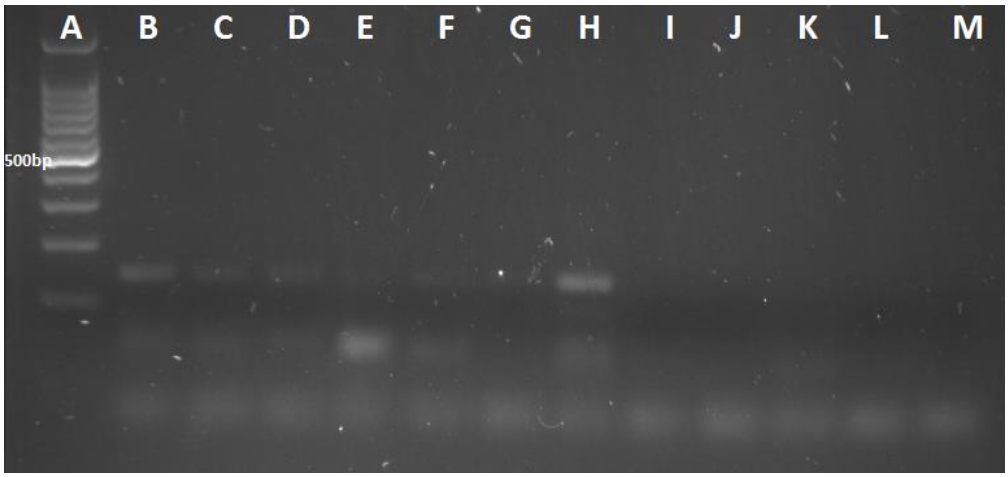


Figure 75: Visualisation of a PCR on a 2% agarose gel to determine the optimal reaction conditions for locus FH127 using pooled Asian elephant DNA. Lane A is 100bp ladder (Promega), and lanes B-M show a temperature gradient in °C of B: 51, C: 51.6, D: 52.6, E: 53.7, F: 54.5, G: 55.5, H: 56.3, I: 57.9, J: 58.6, K: 59.9, L: 61.1 and M: 61.1. The reactions contained 4mM of $MgCL_2$, PCR products of the incorrect size were visualised in lanes B, C, D, E, F, and H, the expected product size was 241bp.

An attempt was made to further optimise the PCR conditions: the primer was reduced from 10um to 0.2um. This resulted in less primer dimer; however, no PCR products were visualised

on a 2% agarose gel at $MgCl_2$ concentrations of 1.5mM, 2mM or 4mM (not shown).

6.3.4.2.11 FH153 PCR

A temperature gradient (55-65^{o}C) and $MgCl_2$ series (1.5mM, 2mM and 4mM) was performed for the locus FH153, a PCR product of the correct size (169bp) was visualised in all lanes, there was no evidence of non-specific binding and low levels of primer dimer (Figure 37). At an $MgCl_2$ concentration of 2mM, faint products of the correct size were visible in most lanes but there was evidence of primer dimer; at a concentration of 4mM multiple PCR products and primer dimer were present (not shown). A primer concentration of 10µm, $MgCL_2$ concentration of 1.5mM and a temperature of 58^{o}C were used in the final reaction.

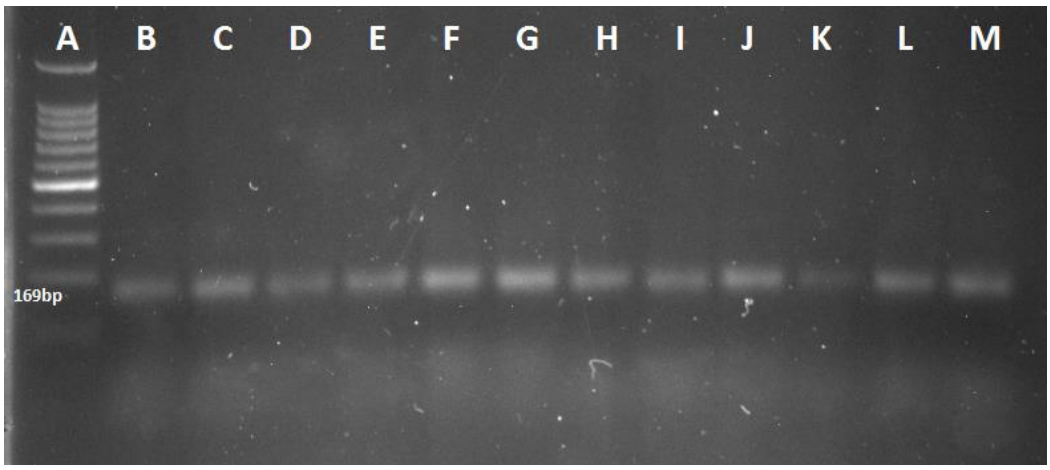


Figure 76: Visualisation of a PCR on a 2% agarose gel to determine the optimal reaction conditions for locus FH153 using pooled Asian elephant DNA. Lane A is 100bp ladder (Promega), and lanes B-M show a temperature gradient in ^{o}C of B: 55, C: 55.6, D: 56.6, E: 57.7, F: 58.5, G: 59.5, H: 60.3, I: 61.9, J: 62.6, K: 63.9, L: 65.1 and M: 65. The reactions contained 1.5mM of $MgCL_2$, a PCR product of the correct size (169bp) was visualised in all lanes.

6.3.4.2.12 LafMS02 PCR

A temperature gradient and three different concentrations of $MgCl_2$, were used to optimise the PCR reaction for loci LafMS02. At a concentration of 1.5mM, products of the correct size (147bp) were visualised in 6 lanes (B, D, E, F, G and H (Figure 38). At a concentration of 2mM products were visualised in all lanes except at a temperature of 57.3^{o}C; products were visible in all lanes at a concentration of 4mM. Primer dimer was visible in all lanes for both concentrations (not shown). A final primer concentration of 0.2µM, an $MgCl_2$ concentration of 1.5mM and a temperature of 54^{o}C were used in the final reaction.

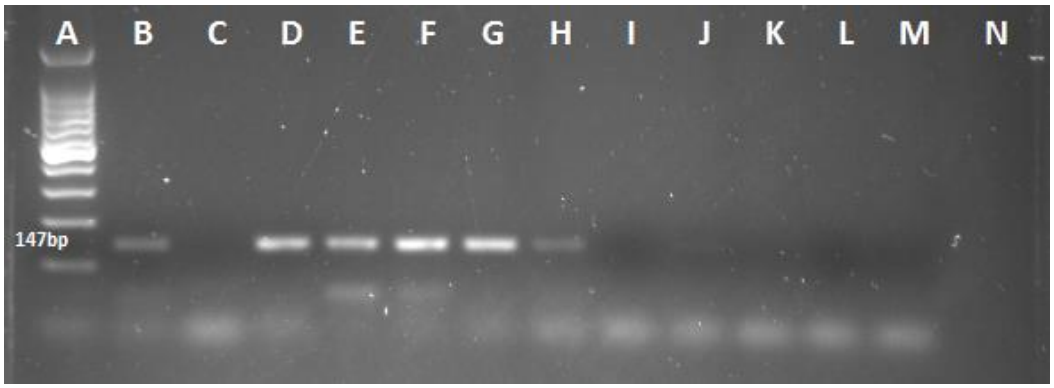


Figure 77: Visualisation of a PCR on a 2% agarose gel to determine the optimal reaction conditions for locus LafMS02 using pooled Asian elephant DNA. Lane A is 100bp ladder (Promega), and lanes B-M show a temperature gradient in degrees C of B: 52, C: 52.6, D: 53.6, E: 54.7, F: 55.5, G: 56.5, H: 57.3, I: 58.9, J: 59.6, K: 60.9, L: 62.1 and M: 62. The reactions contained 1.5mM of $MgCL_2$, a PCR product of the correct size (147bp) was visualised in lanes B, D, E, F, G, and H.

6.3.4.2.13 LafMS03 PCR

Three different $MgCl_2$ concentrations and a temperature gradient were used to determine optimal PCR reaction

conditions. At an $MgCl_2$ concentration of 4mM, products of the correct size (144bp) were visualised in seven lanes (B, C, D, E, F, H and I) (Figure 39). At concentrations of 1.5mM and 2mM no products were visible in any lanes.

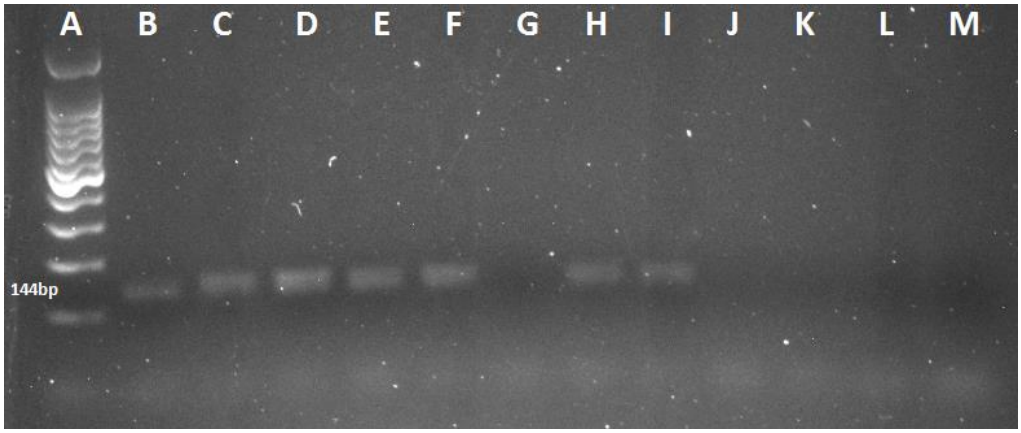


Figure 78: Visualisation of a PCR on a 2% agarose gel to determine the optimal reaction conditions for locus LafMS03 using pooled Asian elephant DNA. Lane A is 100bp ladder (Promega), and lanes B-M show a temperature gradient in degrees C of B: 52, C: 52.6, D: 53.6, E: 54.7, F: 55.5, G: 56.5, H: 57.3, I: 58.9, J: 59.6, K: 60.9, L: 62.1 and M: 62. The reactions contained 4mM of $MgCL_2$, a PCR product of the correct size (144bp) was visualised in lanes B, C, D, E, F, H and I.

6.3.4.2.14 LafMS05 PCR

Optimisation of this locus consisted of a temperature gradient and an $MgCl_2$ series. This resulted in an optimum temperature of 52°C and at an $MgCl_2$ concentration of 1.5mM being selected. Nonspecific binding and smearing was observed in lane C (Figure 40). PCR products were not visualised at $MgCl_2$ concentrations of 2mM and 4mM (not shown).

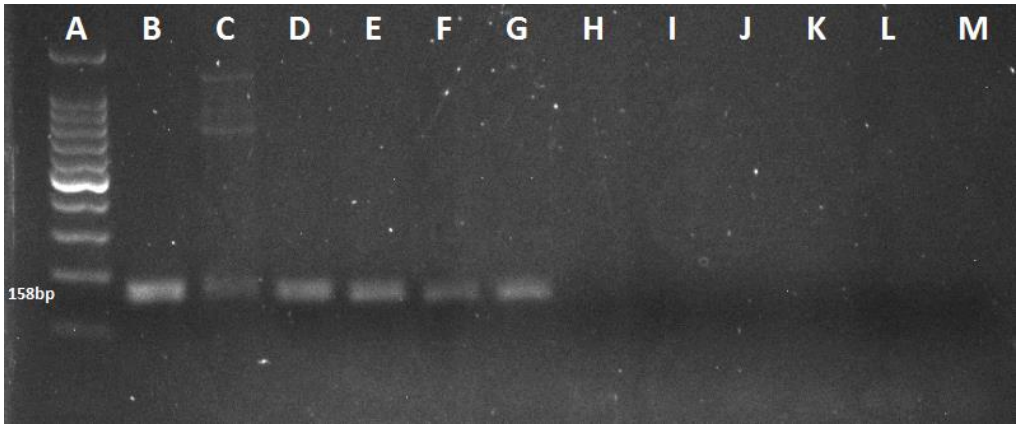


Figure 79: Visualisation of a PCR on a 2% agarose gel to determine the optimal reaction conditions for locus LafMS05 using pooled Asian elephant DNA. Lane A is 100bp ladder (Promega), and lanes B-M show a temperature gradient in ^{o}C of B: 52, C: 52.6, D: 53.6, E: 54.7, F: 55.5, G: 56.5, H: 57.3, I: 58.9, J: 59.6, K: 60.9, L: 62.1 and M: 62. The reactions contained 1.5mM of $MgCl_2$, a PCR product of the correct size (144bp) was visualised in lanes B, C, D, E, F, and G.

6.3.4.3 Final optimisation results

Twelve loci were successfully optimised; after further attempts, optimal conditions were not determined for 2 loci (Table 24).

Table 24: The final working PCR optimal conditions for each successful locus

Loci	Primer concentration	$MgCl_2$ Concentration	Temperature (°C)
EMX-1	0.25µM	1.5mM	62.2
EMX-3	0.25µM	1.5mM	64
LA2	0.2µM	2mM	58
LA4	0.2µM	4mM	54
FH48	10µM	2mM	58
FH60	0.2µM	1.5mM	59
FH94	10µM	1.5mM	61
FH103	10µM	1.5mM	66.6
FH153	10µM	1.5mM	58
LafMS02	0.2µM	1.5mM	54
LafMS03	0.2µM	4mM	54
LafMS05	0.2µM	1.5mM	54
FH102	N/A	N/A	N/A
FH27	N/A	N/A	N/A

6.3.4.4 Fragment length analysis traces

Fragment length analysis was performed on tissue samples from five Asian elephants (UON_1329, UON_1189, UON_1149, UON_1490 and UON_1002). All traces were analysed using GeneMarker, but no samples showed peaks at the expected size. For example, the trace for UON_1329 did not show a peak at the correct size for any of the three loci in the specific well. Three peaks should be visible, one for each locus in the well, EMX-1 labelled with 6-FAM (blue) at 152bp, EMX-3 labelled with HEX (green) at 254bp and LA2 labelled with ROX (red) at 227bp. None of the correct peaks were present in this trace. A red peak was visible in the trace, but it was not at the correct size for the locus LA2 (Figure 41).

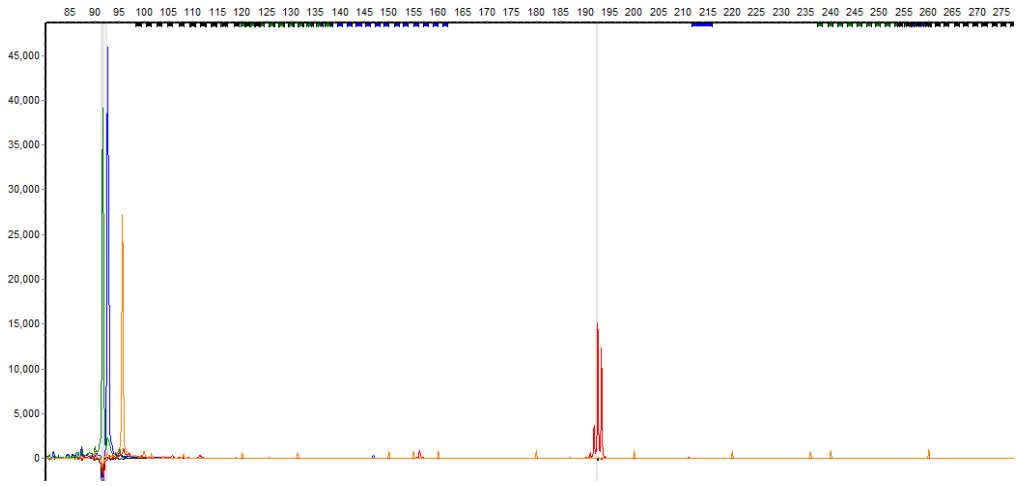


Figure 80: Fragment length analysis trace for UON_1329. The X axis is the height of the peak and the top axis is the size of the peak (bp). Loci in this image should include EMX-1 labelled with 6-FAM (blue) at 152bp, EMX-3 labelled with HEX (green) at 254bp and LA2 labelled with ROX (red) at 227bp. Ladder (LIZ500) shown in orange.

6.4 Discussion

In this preliminary study, we evaluated whether there was any indication of a possible genetic or familial link between EEHV related deaths. A genogram was successfully created and revealed that EEHV deaths appear to group into clusters, inbreeding coefficient data suggests that inbreeding is low within the elephants in this study. However, microsatellite work gave no useful data despite repeated attempts to optimise PCR reactions.

6.4.1 Genogram

The genogram was created to determine initially if a link could be visualised; the data shows that EEHV deaths appear to group in clusters, which suggests there may be a link between

the cases. However, this may suggest either a genetic component or an environmental component, as a number of the clusters were based at the same establishment. The genogram included a total of 39 EEHV related deaths, analysis of these deaths showed a varied geographic location. Twenty-five deaths were reported in the USA and UK with 38.46% (15/39) and 25.64% (10/39) respectively, with the lowest reported number of cases being The Netherlands, Denmark and France at 2.56%, where one case was reported in each country. Underreporting of cause of death and mistaken attribution of death to other causes may contribute to these geographic variations.

6.4.2 Inbreeding coefficients

Inbreeding coefficients were calculated for 445 elephants with known parents.

Values greater than 0.25 or 25% are possible, such as the field spaniel a pedigree dog breed, which in the UK has an average inbreeding coefficient of 0.274 (27.4%), which suggests that dogs of this breed born between January and December 2015 are highly inbred (The Kennel Club (UK), n.d). However, a total mean inbreeding coefficient of 0.009 (0.9%) reported in this study is lower than other captive populations such as captive scimitar horned oryx (Gilbert, 2010), the

studbook reports a mean F value of 0.124 (12.4%), as this is reported as an average it does not mean that all animals are 12.4% inbred. In comparison to wild populations, an F value of 0.009 is lower that F values of wild Baird's tapir (0.11) and the Southern Min Mountains population of wild giant panda (0.05) (Ji-En and Dai, 1994, Norton and Ashley, 2004). In comparison to other captive species, an F value of 0.009 is lower than that of captive Scimitar horned oryx (*Oryx dammah*) with an F value of 0.124. Other animals such as domestic horses report low inbreeding coefficients, including the domestic thoroughbred horse (1.5%) and the domestic quarter horse (0.4%) (McCue et al., 2012) (Table 25).

Table 25: Inbreeding coefficients of selected captive and wild species.

Inbreeding coefficient (F value)	**Species/breed**	**Ref**
0.009	Captive Asian elephant (*Elephas maximus*)	This study
0.274	Domestic dog (Field Spaniel)	The Kennel Club (UK) (n.d)
0.124	Captive scimitar horned oryx (*Oryx dammah*)	Gilbert (2010)
0.15	Domestic thoroughbred horse	McCue et al. (2012)
0.04	Domestic quarter horse	McCue et al. (2012)
0.11	Wild Baird's tapir (*Tapirus bairdii*)	Norton and Ashley (2004)
0.05	Southern Min Mountains wild panda (*Ailuropoda melanoleuca*)	Ji-En and Dai (1994)

As the above describes the average F value of populations, this however does not mean that all animals in the study have an F score the same as the average. In this study a total of 15 Asian elephants presented with an F value of >25% (eight in Europe and seven in North America) and 19 with a value of between 12.5 and 24% (seven in Europe and eight in North America); this suggests that 7.6% (34/445) of the Asian elephants included in the inbreeding study are inbred at 0.125 (12.5%) or above.

There are a number of limitations to both elements of the current study. There may potentially be under reporting of EEHV related deaths, as cases pre-1990 may have been incorrectly attributed to other causes. The reporting of EEHV cases varies by country and institution. There were a limited number of elephants involved in the microsatellite study, as such only a limited number of good quality samples were available; this is a challenge seen in a number of studies investigating EEHV in Asian elephants. There were a number of difficulties in determining optimal conditions for each microsatellite PCR reaction; there are a number of factors that may have contributed to this, including amending several microsatellite PCR reactions, using fluorescent primers instead of radioactively labelled ones, and using different brands of reagents.

The study based on data provided from the genogram sets a baseline for other studies, but may have little relevance to EEHV.

6.4.3 Microsatellites

To further investigate the possibility of a genetic link, microsatellite markers were used to investigate the genetic diversity of Asian elephants located in the UK. Previously

published markers were used to enable a comparison with other populations in range countries. In total 14 loci were selected from previously published studies, 12 loci were used in the final analysis.

To investigate whether non-invasive samples could be used an attempt was made to extract DNA from faeces to be included in the microsatellite analysis. This was unsuccessful as with the addition of the inhibitEX tablet all the supernatant was absorbed. This may suggest a large number of inhibitors were present in the sample, but this may also be due to the samples being stored on Whatman's card, which may not have allowed for an adequate extraction. Similar difficulties were encountered when attempting to extract DNA from elephant faeces directly. Previous studies have used DNA extracted from faecal samples successfully in microsatellite analysis in both African and Asian elephant, and other species including mountain lions, Eurasian badgers (*Meles meles*), and Orang-utans (Ernest et al., 2000, Goossens et al., 2000, Frantz et al., 2003, Vidya and Sukumar, 2005b, Okello et al., 2005, Hájková et al., 2006). However, there have also been reports of difficulties in using faeces for microsatellite studies; Hájková et al. (2006) reported variable success when using faecal samples obtained from wild Eurasian otters (*Lutra lutra*)

for microsatellite analysis. They made a number of recommendations when collecting faeces for microsatellite analysis, including collecting samples during colder months and in the early morning. An attempt was also made to include DNA extracted from trunk washes, whether trunk washes could be used for microsatellite analysis was not established due to time constraints. A number of other non-invasive samples could be used such as plucked hair or skin.

The microsatellite loci selected for this study were chosen as they have been previously shown to amplify in the elephant (Thitaram et al., 2008). However, a number of the loci selected were originally designed for use in the African elephant. We were unable to use two of the 14 loci tested in this study due to optimisation failure; attempts were made to determine the correct temperature and $MgCl_2$ concentration for all reactions. However, this was not successful for loci FH102 and FH127, where further modifications were made in an attempt to obtain a successful PCR, but without success. Reducing the concentration of primers in the reaction was also not successful. In the final analysis of 12 loci, fragment length information was not obtained; PCR reactions were optimised using pooled samples of DNA, however, even with this optimisation most reactions produced a poor yield of PCR

product when visualised. There are a number of reasons a PCR reaction may fail to produce a PCR product including: an incorrect master mix, incorrect concentrations of each reagent, degenerated or poor quality DNA or machine failure (Roux, 1995, Altshuler, 2006, Kennedy, 2011). However, it is surprising that the reactions for all 14 loci were not successful. To overcome this in future experimentation, further optimisation would be needed to ensure good quality bands of the correct size were produced; different reagents such as a proof-reading Taq polymerase could be used.

A number of changes were made to the original PCR protocols for a number of loci; these include changes in primer concentration, $MgCl_2$ concentration or annealing temperature. A comparison of optimisations used for this study, the original publications and those used in another previous study can be seen in Table 26.

Table 26: Optimisations used in the current study compared to PCR protocols in the original publication and a study which also included these loci. Original publication refers to: Nyakaana and Arctander (1998) (LafMS02, 03 and 05), Eggert et al. (2000) (LA2 and LA4), Comstock et al. (2000) (FH48, FH60, FH94, and FH103), Fernando et al. (2001) (EMX-1 and EMX-3) and Comstock et al. (2002) (FH153)

	Primer concentration			$MgCl^2$			Annealing temperature (°C)		
Loci	**This study**	**Original publication**	**Thitaram et al. (2008)**	**This study**	**Original publication**	**Thitaram et al. (2008)**	**This study**	**Original publication**	**Thitaram et al. (2008)**
EMX-1	0.25um	10um	10um	1.5mM	15mM	15mM	62.2	64	60
EMX-3	0.25um	10um	10um	1.5mM	15mM	15mM	64	64	57
LA2	0.2um	0.2um	0.2um	2mM	1.5mM	1.5mM	58	58	62
LA4	0.2um	0.2um	0.2um	4mM	1.5mM	1.5mM	54	54	57
FH48	10um	10um	10um	2mM	2mM	2mM	58	58	58
FH60	0.2um	0.2um	0.2um	1.5mM	2mM	2mM	59	61	61
FH94	10um	10um	10um	1.5mM	2mM	2mM	61	61	61
FH103	10um	10um	10um	1.5mM	2mM	2mM	66.6	58	58
FH153	10um	N/A	N/A	1.5mM	15mM	15mM	58	N/A	50
LafMS02	0.2um	0.2um	0.2um	1.5mM	1.5mM	1.5mM	54	51	48
LafMS03	0.2um	0.2um	0.2um	4mM	1.5mM	1.5mM	54	50	50
LafMS05	0.2um	0.2um	0.2um	1.5mM	1.5mM	1.5mM	54	49	48

Overall the results from this study do not provide enough evidence to suggest there is a genetic or familial component to EEHV. Microsatellite loci were not able to be detected, but a significant limitation of this study is that DNA was only successfully extracted from five Asian elephants, more samples would be needed to enable data obtained to be significant.

Future work would include building on the current study; a greater number of elephants could be assessed which had tested either negatively or positively for EEHV (so that EEHV status was known for each elephant). As recent studies have shown HHV6 in humans can be transmitted from parent to child due to chromosome integration, and Gallid herpesvirus 2 (GaHV2) the causative agent of Marek's disease in chickens is also able to integrate into the chromosomes (Delecluse and Hammerschmidt, 1993, Tanaka-Taya et al., 2004, Osterrieder et al., 2014), it is reasonable to consider that this may also be possible for EEHV. The possible heritability and whether EEHV integrates into the chromosomes of Asian and African elephants has not yet been investigated. This could be done by harvesting metaphase cells and then a chromosome spread. The technique has previously been used to determine the location of viral integration sites on the chromosomes for

adenovirus and hepatitis B virus (Recchia et al., 1999, Huang et al., 2002a). It has also been used to determine integration sites of two herpesviruses, GaHV2 and HHV6 (Delecluse and Hammerschmidt, 1993, Daibata et al., 1999).

In conclusion the genogram created in this study may be used for further studies. Microsatellite analysis may be a useful tool in other studies, but techniques need further optimisation.

Chapter 7 – The use of microarrays to detect potential co-pathogens and EEHV

7 The use of microarrays to detect potential co-pathogens and EEHV

7.1 Introduction

It is not uncommon for humans and other animals to be infected with more than one pathogen at a time; these are referred to as co-pathogens. Many co-infections do not cause any extra problems for the patient; however, some can cause complications. Several studies have suggested that HSV infection is facilitating the spread of HIV, as patients with HSV are more susceptible to acquiring HIV (Augenbraun et al., 1995, Mole et al., 1997, Weiss et al., 2001, Rodríguez et al., 2002, Corey et al., 2004, Brown et al., 2007).

The exact mechanism of how HSV2 increases susceptibility to HIV is currently unknown, however, Martinelli et al. (2011) describe the possible involvement of dendritic cells in the process. Dendritic cells are the site of entry, enabling spread to the mucosa for both HSV2 and HIV1. Retinoic acid, produced by specialized dendritic cells in the gut, is an immunomodulator which influences the replication of HIV-1 and mediates integrin α4β7 expression on lymphocytes. An increase in α4β7 expression was seen on $CD4^+$ cells. The study suggests HSV2 is able to modulate its environment, this

influences the function of dendritic cells, potentially amplifying $\alpha4\beta7^{high}CD4^{+}$T cells which is a highly susceptible HIV1 target cell, playing a role in susceptibility to HIV1 infection.

It has been suggested that promoting treatment of those infected with HSV2 may contribute to reducing the transmission of HIV (Webster, 2007). To determine if treating HIV positive/HSV2 positive patients with antivirals reduced the levels of HIV1 RNA, Schacker et al. (2002) administered high doses of acyclovir and measured HIV-1 RNA loads at regular intervals. A strong correlation was observed between total HSV shedding rate and plasma HIV1 RNA loads. When acyclovir was administered HIV1 RNA levels were 48% lower in comparison to no HSV treatment. The study suggests daily treatment for HSV infections may be important in the management of HSV/HIV positive people.

Co-infections have also been seen in animals. Post weaning multisystemic wasting syndrome (PWMS) seen in pigs, is clinically characterized by pallor of the skin, poor body condition, jaundice and dyspnoea (Allan and Ellis, 2000, Kim et al., 2002). The viral agent associated with PMWS is porcine circovirus 2 (PCV2). Infection with the virus by itself usually only causes mild disease (Ellis, 2014), however, the presence

of co-pathogens such as porcine parvovirus can lead to increased replication of PCV2, which induces more serve clinical disease and lesions (Ellis et al., 2000, Kennedy et al., 2000).

Most clinical cases of EEHV in Asian elephants have not been investigated for the presence of potential co-pathogens, and the role of co-pathogens in clinical cases of EEHV has not been established.

7.2 Emerging diseases

An emerging infectious disease (EID) is one which appears for the first time in a population, or which is rapidly increasing in geographic range or prevalence (Morse, 1995, Morse, 2001). There are three tiers of emerging pathogens, tier 1 (those transmitted from animal to animal), tier 2 (those that are able to opportunistically infect humans) and tier 3 (pathogens that can be transmitted from human to human without the involvement of an animal host) (Rosenberg, 2015). EID's have occurred throughout history, with disease spreading through translocation. Translocation is the geographic movement of animals and people into new areas; this can potentially introduce pathogens into new regions, or expose animals or humans to new pathogens. (Guris et al., 2008). Diseases spread in this way include the Black Death (plague), which

was brought to Europe by ship from Asia, and measles and smallpox, which were introduced into the Americas from Europe by Spanish conquistadors (Siedler and Arndt, 2010, Malik and Roy, 2008). EID's can present a risk to livestock, which can lead to economic loss through the death of saleable livestock. An example of this is African swine fever, which was introduced into Europe in 2007, when an outbreak occurred in Georgia, spreading amongst domestic pigs and wild boar (Rowlands et al., 2008). Other factors may influence the spread of EIDS, including climate change; this may increase the geographic range of arthropod vectors. The transmission season for certain diseases may also be extended due to climate change, an increase in temperature could mean vectors such as *Aedes aegypti,* the vector for dengue, could establish in Southern Europe (Huang et al., 2002b).

7.3 Monitoring and surveillance

Monitoring an outbreak can be difficult as it usually takes place when an outbreak has occurred, and may not play a clear role in the detection of emerging disease as it is usually cryptic and spontaneous.

There are a number of monitoring, surveillance and prediction methods that have been used to look at emerging diseases and outbreaks. The goal when using indicator based evidence

is to find increases in cases or clusters of cases that may indicate a threat. Indicator based data can be collected from various sources including reports from diseases requiring mandatory notification, vector surveillance, mortality data, hospital/vet admissions, and pharmacy monitoring (Velasco et al., 2014). Data may also be collected from field clinical observations, reports from clinicians and information from agriculture and industry such as epidemiology and risk factors (Meerburg et al., 2009, Woolhouse, 2011, Humblet et al., 2012, Velasco et al., 2014)

There are a number of new technologies that have been used to monitor and predict emerging infectious diseases. Internet based surveillance is through event-based evidence and includes a diverse range of resources including social media, news reports, forums and internet searches (Milinovich et al., 2014). Event-based surveillance systems are present on the internet and include Program for Monitoring Emerging Disease (ProMED mail) and the Global Public Health Intelligence Network (GPHIN) (Yu and Madoff, 2004, Mykhalovskiy and Weir, 2006). A large number of news items are reported through these systems each day, however, not all the data and reports are able to be investigated and verified (Velasco et al., 2014). Models can be useful in predicting epidemics and

disease spread, Infectious disease modelling enables an epidemic simulation, this allows for potential patterns and the progression of epidemics to be studied (Christaki, 2015). The Global Epidemic and Mobility model is widely used as an infectious disease model, it has been used for the modelling of influenza, taking into account demographics and air travel (Christaki, 2015).

Monitoring and surveillance tools can be useful in the prediction and prevention of emerging infectious disease however, this requires a large amount of data analysis and investigation which may not always take place. Even with these tools in place, it is difficult to predict and monitor potential outbreaks.

7.4 The relationship of rodents and spread of disease

There are more than 2000 species of rodents, which account for 44% of all mammal species (Wolff and Sherman, 2007). Rodents have been associated with the transmission of diseases to both humans and animals (including pets, domestic animals and wildlife) (Frean et al., 1991, Frenkel et al., 1995, Gratz, 1999, Davis and Calvet, 2005, Lilenbaum et al., 2005, Kijlstra et al., 2008, Backhans and Fellström, 2012).

They can act as a liaison host which is a species that acts as a link in the chain of transmission between the reservoir host and their definitive host. The liaison host may or may not show signs of disease. This suggests rodents can transmit pathogens from a reservoir species to others (Garnham, 1971). It is common for pests such as rodents to live in close contact to other animal species, including exotic species in captive environments (Forthman and Ogden, 1992). This has potentially facilitated the spread of diseases in zoo environments. One example of this is an outbreak of cowpox virus. An outbreak of cowpox virus occurred in a UK zoo in 1977, which resulted in the death of three Cheetahs. The source of this outbreak was undetermined but it was suggested that it may have been spread by rodents (Baxby et al., 1982). No similar outbreaks had been reported previously in the UK, however, rodents were suggested as a source of infection due to a similar outbreak in Moscow caused by the use of infected white rats used as food for captive cheetahs (Baxby et al., 1982). There is also evidence that pathogens from rodents can be spread to elephants in captive environments. One study reported the transmission of cowpox from rat to elephant, and subsequently from elephant to human (Kurth et al., 2008). It has been suggested that in

order to predict the emergence of disease, the surveillance of rodents is important (Meerburg et al., 2009).

7.5 Pathogens for investigation

This study has been conducted to explore the possible presence of a large number of pathogens, transmitted by rodents which might potentially infect elephants in zoos. Using a microarray that has been previously been designed to detect a number of pathogens present in rodent populations. Pathogens were chosen to be included on the microarray based on: (1) their similarity to EEHV in their clinical presentation in elephants, and (2) whether they could potentially be present in the local rodent population resident in zoos.

Encephalomyocarditis virus was included as a pathogen in this study as animals with this virus present with similar clinical signs to those with clinical EEHV. EMCV has been reported in both captive and wild populations of African and Asian elephants; clinical signs include anorexia, dyspnoea and listlessness, but the most common presentation is sudden death (Simpson et al., 1977). It has been suggested that EMCV is transmitted to elephants via infected rodents, several studies have investigated rodent populations to understand more about the prevalence of EMCV. A study by Reddacliff et

al. (1997) found only one positive rodent out of a total of 54 rodent samples (1.8%), however, Billinis (2009) was not able to isolate EMCV from 42 brown rats but 35 were seropositive suggesting a previous exposure. In comparison a more recent study found a prevalence rate of 9% (11/125) in rodents captured from Swedish pig and chicken farms (Backhans et al., 2013). However, lower prevalence rates have been reported in other locations, Truong et al. (2013) investigated the prevalence of EMCV in 102 rodents from areas surrounding pig farms in South Korea, 3.9% (4/102) tested positive by PCR. The main route of transmission of EMCV is contamination of food/water supplies with faeces from infected rodent and/or eating of infected rodents (Maclachlan and Dubovi, 2011). Reducing risk factors such as contact with infected rodents, contaminated food/drink and contact with infected faeces could reduce the amount of transmission (Maclachlan and Dubovi, 2011).

EMCV has been identified as a cause of major production losses in intensive piggeries. The virus tends to cause explosive sporadic outbreaks, which are potentially the result of transfer from rodents indirectly due to food/drink contamination with rodent faeces or ingestion of rodent carcasses (Dea et al., 1991). It is also thought that pig to pig

transmission may be possible, this has been investigated in an experimental laboratory setting by placing an infected animal in close contact with non-infected animals, there is no mention in the study if this has also been seen in none experimental animals (Maurice et al., 2002). The estimated R_0 in the pig to pig transmission study was around 1, this would suggest that the spread of EMCV pig to pig is limited but may also lead to larger outbreaks (Maurice et al., 2002). Previous EMCV studies also found transmission of EMCV from pig to pig via a contact animal (Foni et al., 1993, Billinis et al., 1999). Non-human primates also appear to be highly susceptible to the virus; EMCV fatalities have been reported in the ring-tailed lemur (*Lemur catta*), black lemur (*Eulemur macaco*), white-headed lemur (*Eulemur albifrons*), red-ruffed lemur (*Varecia rubra*), common marmoset (*Callithrix jacchus*), Barbary macaque (*Macaca Sylvanus*), squirrel monkey (Saimiri spp), DeBrazza's guenon (*Cercopithecus neglectus*), baboon (*Papio spp*), mandrill (*Mandrillus sphinx*) and chimpanzee (*Pan troglodytes*) (Gaskin et al., 1980, Reddacliff et al., 1997, Canelli et al., 2010). Clinical signs in primates include lack of coordination, anorexia, sensory depression, lethargy, and sudden death (Reddacliff et al., 1997).

EMCV has occurred in mass outbreaks in wild populations of African elephants, with 64 elephants dying in the Kruger National Park during one such outbreak (Grobler et al., 1995). Necropsy samples from this outbreak showed myocarditis lesions; other observations made during necropsy include a mottled, streaky myocardium, congested lungs, swollen liver and brain congestion. EMCV was thought to be the cause due to the presence of cardiac lesions (Grobler et al., 1995). Similar post mortem findings such as lesions of the heart and congestion of the lungs have since been observed in other species (Billinis et al., 1999). Specimens from three elephants were positive for EMCV when tested with PCR (Grobler et al., 1995). Four captive African elephants died from EMCV infection in years 1976 and 1977 in the USA (Simpson et al., 1977), whereas the first reported case in a captive African elephant in Europe occurred in 2013. The 18 year old female was found dead without any prior clinical signs; a post mortem examination reported lesions and haemorrhages on mucosa of the eyes, mouth, rectum and vagina. Lesions were also reported on a number of internal organs; EMCV was then isolated from samples obtained from the infected organs using BHK-21 cells (Lamglait et al., 2015). Gaskin et al. (1980) also reported a suspected case of EMCV in an Asian elephant at a zoo in Florida. The animal, aged 10, died suddenly in 1975.

Necropsy findings showed myocarditis and a flaccid heart, as well as haemorrhages in the lungs, kidneys and intestine.

A number of other pathogens can be transmitted to elephants, both from the environment and from other animals. These pathogens include *Mycobacterium spp,* which include Mycobacterium tuberculosis and *M. bovis*. If an elephant is infected with either of these species, this may lead to disease, however, most animals including elephants with an active TB infection show no clinical signs (De Lisle et al., 2002). In cases of TB infection in elephants, clinical signs may include weight loss, anorexia, weakness, coughing and nasal discharge (Pinto et al., 1973, Gutter, 1981, Saunders, 1983, Angkawanish et al., 2010). Tubular lesions are also seen in elephants, their appearance varies depending on the stage of the disease; granulomatous lesions can be observed in the bronchial lymph nodes and pulmonary tissue. In >66% of cases where both lungs are involved, caseocalcareous and cavitating lesions have also been observed; this can then result in the presence of large pulmonary abscesses (Mikota et al., 2000).

There are a number of treatment protocols for mycobacterial infections in elephants available suggesting both first line and second line drugs. First line drugs include isoniazid,

pyrazinamide, rifampin, ethambutol and streptomycin, and second line drugs include amikacin, ethionamide, quinolones and capreomycin, however, there may be multidrug resistance (Dumonceaux and Mikota, n.d).

Anti-tuberculosis treatments are known to affect the performance of serological assays, a treated elephant may appear positive by Elephant tuberculosis Stat-Pak but will eventually become negative (Lyashchenko et al., 2006).

Tuberculosis from elephants can also spread to other animals including humans. This could potentially present a major health concern for zoo keeping staff and members of the visiting public.

A number of other pathogens that have not been previously reported in Asian elephants were also included in this investigation. These include *Rickettsia spp*, lymphocytic choriomeningitis (LCMV), Puumala Hantavirus and Seoul Hantavirus. By contrast, *M. tuberculosis*, *M. bovis*, *Leptospira interrogans*, cowpox virus, *Yersinia spp*, *Toxoplasma gondii*, EEHV and EMCV, which were included in the pathogens to be investigated, have been reported in elephants previously.

7.6 Assay for the detection of multiple pathogens

Being able to detect a number of pathogens simultaneously can be less time consuming than testing for each pathogen individually, and provide a more rapid diagnosis. When a number of pathogens are present such as the presence of HIV and *T.gondii*, it can lead to more serious conditions and complications. To investigate the presence of multiple pathogens or multiple species, a number of different types of testing methods can be used.

7.6.1 TaqMan Array Card (TAC)

The TaqMan Array Card (TAC), a singleplex real-time PCR assay, can detect multiple pathogens simultaneously, including a mixture of both DNA and RNA pathogens (Kodani et al., 2011, Rachwal et al., 2012, Liu et al., 2013, Weinberg et al., 2013). Samples are added to the channels on the card, depending on the experiment the cards contain a number of channels. Rachwal et al. (2012) used a TAC with 8 channels allowing for 24 individual reactions to allow the detection of multiple biological agents. There are a number of limitations to this type of assay, it is expensive; even when only one or two pathogens are of interest, the whole card must still be used. Furthermore, duplicates are needed, especially in the case of low level infections due to sensitivity. However, such assays

are useful as it allows broad range screening (Eisen and Gage, 2009).

7.6.2 Next generation sequencing (NGS)

Next generation sequencing allows for the detection of any nucleic acid present in a sample. The most common method of NGS is illumina; this has also been used for pathogen discovery. Vayssier-Taussat et al. (2013) found bacterial pathogens in ticks in Western Europe using NGS. RNA was extracted from 1450 *Ixodes ricinus* nymphs located in France, RNA was then pooled and used for NGS. The study identified 12 different bacteria species including *Candidatus neoehrlichia mikurensis* which had not been previously reported in France. The main advantage to using NGS is the production of large amounts of DNA/RNA sequences, which can then be analysed by a bioinformatician and the species identified. This allows for the identification of everything present in the sample, useful for pathogen discovery. It is also very sensitive and can detect the DNA from pathogens at low levels. The main disadvantages include the need for specialised equipment and the cost, also the need for specialised bioinformaticians to analyse data (Barzon et al., 2013). Using NGS may be useful in the detection of multiple pathogens in a single sample, or for pathogen discovery in multiple animal samples, it may also

be used in the diagnosis of infectious disease (Vayssier-Taussat et al., 2013).

7.6.3 Multiplex PCR

Conventional PCR assays can be developed to allow the detection of multiple pathogens in one assay. Multiplex PCR allows for a number of pathogens to be detected in a sample using primer pairs optimised for each pathogen. A limitation of this method is that it can be difficult to combine all the primer pairs into a working reaction. This may be due to differences in optimal annealing temperatures and reaction conditions; there may also be poor sensitivity or specificity, increased formation of primer dimers, and non-specific binding. A compromise of poorer sensitivity and reduced specificity may be made to enable multiple pathogens to be detected simultaneously in comparison to singleplex PCR (Koenen et al., 1999, Carocci and Bakkali-Kassimi, 2012). Multiplex PCR has been used to detect a number of viral pathogens, Elnifro et al. (2000) reported the used of multiplex PCR to investigate the presence of HSV1, HSV2, CMV, enterovirus, echovirus type 22, LCMV, mumps, and measles. This study reported that in controlled testing, the sensitivity of this multiplex PCR was no different to that of the reactions using single primer pairs. This would suggest that a number of pathogens can be detected with no compromise to sensitivity.

7.6.4 Real-time PCR

Real-time PCR is a technique that detects the accumulation of amplicons in real time, detecting amplicons during the exponential phase enables quantification, in comparison to conventional PCR where products are detected during the plateau phase at the end of the reaction. Real time PCR has been used for the rapid detection of multiple pathogens. Lehmann et al. (2008) used this type of assay to detect 25 different type of fungi and bacteria in human blood. Blood samples were mechanically lysed using ceramic beads; DNA prepared using a commercial kit. PCR amplification was then performed using a LightCycler. With the addition of a melt curve at the end of the amplification individual bacterial and fungal species can be identified as their melting points differ. The advantage of using this type of assay is that it is rapid compared to NGS, and has an increased sensitivity compared to conventional PCR. However, it does require expensive equipment compared to conventional PCR.

7.6.5 Microarrays

7.6.5.1 Overview

Microarrays can be used as a diagnostic tool that allows samples to be screened for multiple pathogens. A microarray platform contains an arrangement of sequence-specific DNA

sequences (probes) attached to a solid surface such as a glass slide, silicon chip, nylon membrane or tubes, usually configured in rows and columns (Bertucci et al., 1999, Lenigk et al., 2001, Heller, 2002, Wang et al., 2003, Giles et al., 2015). Complimentary DNA or cDNA within a sample are hybridised to the probes, non-specific sequences are then washed off, and then only strongly bonded sequences remain hybridized. The total strength of the signal from each hybridised spot on the surface is measured, the amount of target sample that has hybridised to the probe determines the strength of the signal (Blalock, 2003, De Rinaldis and Lahm, 2007, Nuber, 2007, Wiesinger-Mayr et al., 2007).

7.6.5.2 Probe development

Probes designed for use in a microarray experiment should be designed to be specific to their target, this increases binding efficiency. If the probe is not specific, then cross-hybridization may occur. The sensitivity of the probe is also important; they should bind to their target even when there is a low abundance of the target sequence. Repeated experiments should be conducted to determine probe hybridisation is consistent (Loy and Bodrossy, 2006, Giles et al., 2015). Short or long oligonucleotide sequences can be designed as probes, however, longer sequences (50-70bp) may decrease the specificity of the array, when specificity is lowered, sequences

with reduced homology may be detected (eg. Cross-species). Longer probes are preferred in some circumstances, including when there is not an existing array available, when detecting similar species may be beneficial, this would include the detection of novel species/pathogens (Bar-Or et al., 2007). However, there are also circumstances when short oligonucleotide sequences of 15-30bp, are useful as it allows the probe to be highly species-specific. Short sequences may be useful in strain identification (Tomiuk and Hofmann, 2001, Wang et al., 2003, Liu et al., 2010). Array sensitivity is determined by the intensity with which the probe binds to its target sequence (Lemoine et al., 2009). Probe sensitivity is affected by: the concentration of the probe on the surface, thermodynamic properties of each probe, and the availability of the target sequence (Draghici et al., 2006). The hybridisation process may also be affected by certain factors which may affect the annealing of the probe with the target sequence, including the concentration of salt and temperatures, used a series of washes ensures that any non-specifically bound DNA is washed away from the array surface. A larger amount of wash buffer is used in comparison to the amount used during the hybridisation step, this is to ensure impurities and contaminants are diluted. Washing reduces the signal caused by any impurities, as they may contribute to the

amount of background noise (random signal variation) (Russell et al., 2008, Giles et al., 2015, Giles, 2015).

7.6.5.3 Microarray variability

There may be an amount of variation in microarray experiments, these variations may occur for a number of reasons, including small changes in sample labelling or processing. Including replicates may reduce some variation. The probes attached to the array, may also be a source of variation, it is thought that by optimising both the array and the hybridisation process this can be reduced (Russell et al., 2008). Including replicates allows variation to be measured; however, this is not always done. There are disadvantages to microarray studies without replicates, such as lower sensitivity and biological variation (Lee et al., 2000).

7.6.5.4 Multiple pathogen detection

Microarrays can be for the detection of pathogens in animals, including the simultaneous detection of Newcastle disease and avian influenza in birds (Wang et al., 2008). The use of microarrays has been valuable in screening rodents for the presence of zoonotic pathogens; an assay developed by Giles et al. (2015) was used to detect 17 pathogens of rodents simultaneously in a sample. Early detection of pathogens may

allow for a quicker response and prevention of further spread of disease.

7.6.5.5 The detection of multiple herpes viruses

Microarrays have been used to detect several human herpes viruses (Földes-Papp et al., 2004, Jääskeläinen et al., 2006, Zheng et al., 2008). Jääskeläinen et al. (2006) investigated samples of cerebral spinal fluid, which were screened for the presence of eight human herpes viruses. Target pathogens included HSV1, HSV2, CMV, EBV, HHV7, HHV6A, HHV6B and VZV. A small number of samples (10/227) tested positive for a concurrent infection with two viruses and one sample presented with 3 viruses. A later study by Zheng et al. (2008) used amino modified glass slides spotted with a number of probes for detecting viral DNA. Pathogens which could be detected included HSV1, HSV2, VZV, EBV, CMV, HHV6A and HHV6B. One hundred and thirty two blood samples from children were tested, of which 49 were positive for herpes virus. Five presented with concurrent infections, two EBV and CMV, one VZV and CMV, one HSV2 and CMV, and one EBV and HHV-6B.

A study by Giles et al. (2015) described the first development of an alternative to the glass slide microarray (as this can be expensive). The two array platforms used in the study were

the ArrayTube (AT) platform and the ArrayStrip (AS) platform both from Alere Technologies GmbH (Jena, Germany). The AT allows up to 225 probe spots whereas the AS platform allows up to 600. The probe spots are located on a small array surface 4 x 4mm which is located at the bottom of a vial.

7.6.5.6 Advantages

There are a number of advantages and disadvantages of using microarrays. They are useful in mutation analysis, genotyping and detecting bacteria, viruses and parasites (Kumar, 2009). Using this technology a large number of samples can be screened for multiple pathogens, in comparison to PCR where screening large numbers of samples may be difficult (Cannon et al., 2010). Previous studies have suggested that microarrays are semi-quantitative, if there is more target DNA/cDNA within the sample more hybridisation occurs and so a stronger signal is then emitted, microarray results can be compared to those from qPCRs (Chuaqui et al., 2002, Dallas et al., 2005). More recent gene transcription studies have also shown a good correlation between microarray results and qPCR, further suggesting the semi-quantitative use of microarrays (Kouni et al., 2013, Martínez et al., 2015).

7.6.5.7 Disadvantages

There are also a number of disadvantages in developing and using a microarray system. The main disadvantage of glass slide microarray systems is cost, however, an alternative ArrayTube has been developed which uses less specialised equipment and is more cost effective (Ehricht et al., 2006).

There can be difficulty in appropriate probe selection, which can be a time consuming process. It may also be difficult to design probes that are specific to a single organism. Probes need to be chosen based on their sensitivity, specificity, high coverage to include all strains needed in the experiment, however, cross-hybridisation is more likely in sequences that provide high coverage (Tomiuk and Hofmann, 2001, Liu et al., 2010). A number of factors can affect the hybridisation process, including probe length and hybridisation temperature, hybridisation process optimisation may also be time consuming. A number of considerations must be included during the optimisation process such as, probe length, hybridisation temperature and preparation of the target (Schena, 2003). Sensitivity of the assay may also be lowered by the presence of nucleic acid from the host within the samples, however, there are specialised commercial kits

available which can be remove the host nucleic acid prior to the initial PCR amplification (Gurrala et al., 2009).

7.6.6 Conclusion

Microarray technology has not previously been used to screen elephants for multiple pathogens. The current study focused on pathogens that are considered zoonotic and are commonly found in rodents, as rodents may be present in captive elephant environments and able to transmit disease. By using an array system developed for screening rodents, the same system can be used to screen Asian elephants for potential rodent borne pathogens. With further modification of the array it could also be used for screening EEHV.

Screening rodents is one method for investigating the prevalence of pathogens in a specified area. Screening rodents in a zoo setting is important for the health of exotic captive species, zoo keeping staff and members of the visiting public. Early detection of pathogens may help to prevent a potential outbreak.

7.7 Materials and methods

7.7.1 Samples

A total of 18 clinical tissue samples from Asian elephants, and trunk wash samples (for sample collection see materials and

methods 2.1) from nine Asian elephants, were investigated for the presence of multiple pathogens. Tissue samples were provided from 4 animals that had previously tested positive for EEHV using qPCR, and one which tested negative (Table 27). RNA and DNA was obtained from tissue, and where tissue was not available, previously extracted DNA was used.

Table 27: List of tissue samples used to investigate the presence of multiple pathogens.

Animal ID	Tissue	Nucleic acid sample
UON_1329	Heart	DNA/RNA
UON_1329	Liver	DNA/RNA
UON_1329	Lung	DNA/RNA
UON_1329	Kidney	DNA/RNA
UON_1189	Heart	DNA/RNA
UON_1189	Liver	DNA/RNA
UON_1189	Lung	DNA/RNA
UON_1189	Kidney	DNA/RNA
UON_1002	Heart	DNA
UON_1002	Liver	DNA
UON_1002	Lung	DNA
UON_1490	Liver	DNA
UON_1490	Lung	DNA
UON_1490	Kidney	DNA
UON_1149	Heart	DNA/RNA
UON_1149	Liver	DNA/RNA
UON_1149	Lung	DNA
UON_1149	Kidney	DNA/RNA
UON_1140	Trunk wash	DNA
UON_1625	Trunk wash	DNA
UON_1089	Trunk wash	DNA
UON_1093	Trunk wash	DNA
UON_1175	Trunk wash	DNA
UON_1309	Trunk wash	DNA
UON_1579	Trunk wash	DNA
UON_1543	Trunk wash	DNA
UON_1353	Trunk wash	DNA

7.7.2 DNA extraction

See materials and methods section 2.2.1

7.7.3 RNA extraction

RNA was extracted from tissue samples using a commercial kit (Qiagen RNeasy mini kit) and following the manufacturer's instructions. Tissues were initially lysed using a tissue lyser (Qiagen TissueLyser II), set at 30Hz for 2 minutes using steel beater beads. The supernatant was then used for the extraction, 70% alcohol and the supernatant were transferred to a spin column, then centrifuged for 15 seconds at 8000xg. The membrane was then washed using a series of three solutions, the sample was then eluted in 50µl RNase free water, and stored at -20^{o}C until use.

7.7.4 EMCV positive control

Positive control material (cDNA) was a cell culture adapted strain ATCC® VR-129™ obtained from The Veterinary and Agrochemical Research Center, Belgium.

7.7.5 Microarray

7.7.5.1 Pathogens

The array system in this study was used to detect a series of bacterial and viral pathogens (Table 28). Some of the pathogens have previously been detected in rodents within zoos in the UK. These include *Mycobacterium spp*, *L.*

interrogans, *Rickettsia spp*, LCMV, Cowpox virus, *Hantavirus spp* and *Yersinia spp*. EMCV was selected due to a similar pathogenesis to EEHV in elephants.

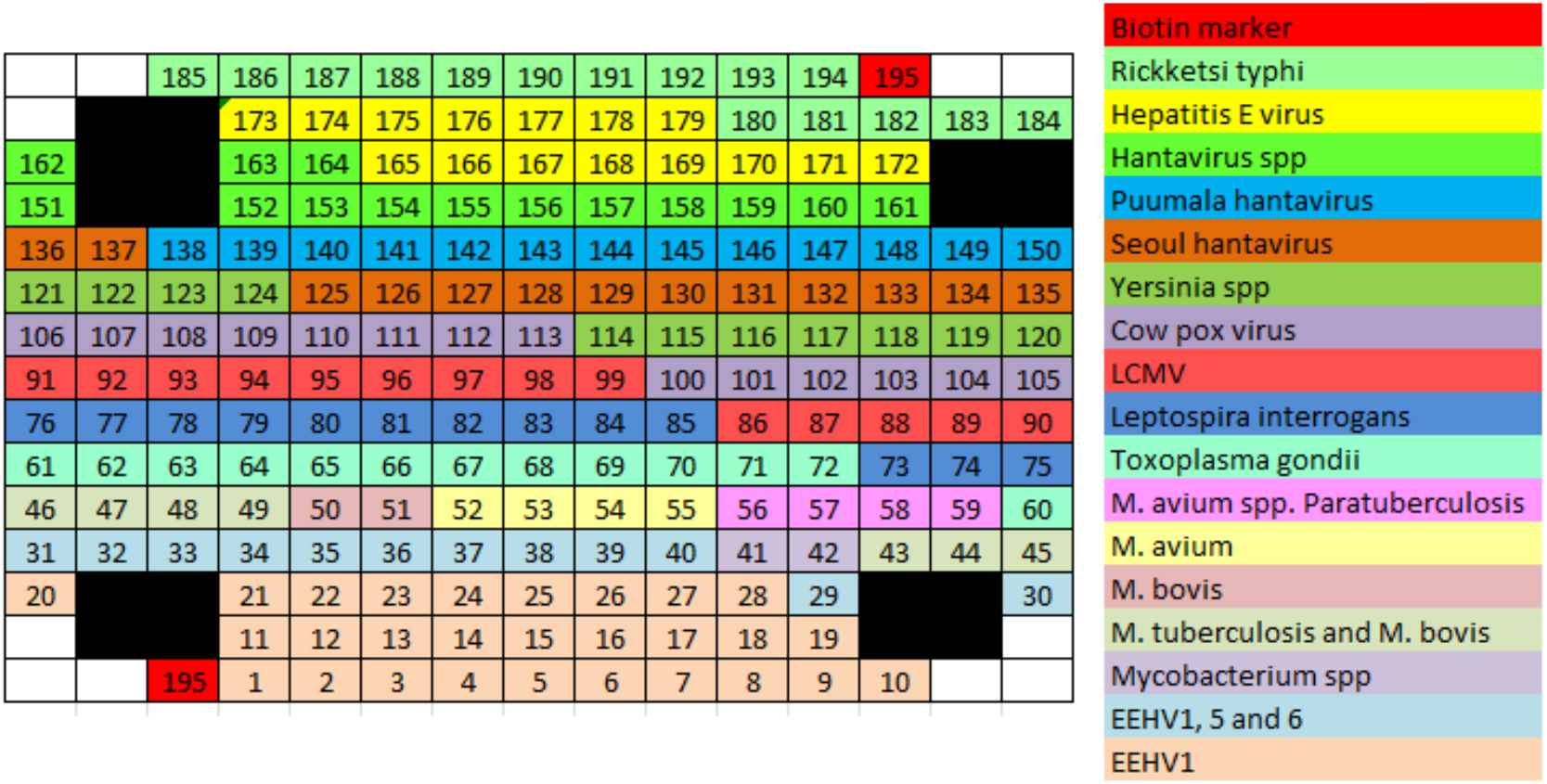
Table 28: Pathogens targeted using the ArrayTube (AT) system. A number of bacterial and viral targets were used in the microarray. EEHV and EMCV probes were also added to the array.

Type	Pathogen
Bacteria	Mycobacterium tuberculosis
	Mycobacterium bovis
	Mycobacterium spp
	Leptospira interrogans
	Rickettsia typhi
	Rickettsia spp
	Yersinia spp
Virus	LCMV
	Cowpox virus
	Puumala hantavirus
	Seoul hantavirus
	Hantavirus spp
	EEHV
	EMCV

7.7.5.2 Layout

The screening chip had a total of 196 spots, with spot 195 (biotin marker) repeated twice, in the top right and bottom left corners (Figure 42).

Figure 42: A colour coded layout of the array tube chip. All probes appear once on the chip with the exception of the biotin marker (spot 195). Black areas were orientation markers. As a number of pathogens on this chip we used previously to screen rodents; Hepatitis E virus was excluded from these experiments, as this has never been reported in elephants.



7.7.6 Working probes

Multiple probes were used for each set of primers, a number of probes were confirmed to hybridise positively in a previous study by Giles et al (Giles et al., 2015), primers and probes used are as stated in the study and are highlighted in red text in Table 29. Those highlighted in blue in the same table were also from this previous study, though positive control material was not available, this meant their hybridisation status was not confirmed. Those highlighted in purple text were developed for this study; positive control material was available for both EEHV1 and EMCV to confirm the probes hybridise.

Table 29: Primer sequences and working probes list.

Forward primer (5" to 3" sequence)	Reverse primer (5" to 3" sequence)	Number of working probes*	Predicted target
TGCACTTCGGGATAAGCCTG	TAGCATGTGTGAAGCCCTGG	2	Mycobacteria broad spectrum
CAACGGCCTCAACCTCGTC	CATTGAAAATGGCCCCACCG	2	*M. tuberculosis, M. bovis, M. canetti* and *M. africanum*
AACGCGCTRTCCACCCGGCT	GTGATGATCGCCTGRACTTCGGTG	6	*M. tuberculosis, M. bovis, M. canetti, M. africanum, M. caprae* and *M. microtti*
CATGAACGGAGCGCATCAC	CGGAGCGGGGATGTAATAGG	4	*M. avium* and *M.avium ssp paratuberculosis*
TTGGCGGCGACAAAGTTAAT	TTCGTCTGTCAAGCAACGGA	4	*M. avium* and *M.avium ssp paratuberculosis*
CTCCGTCGTCCGTCGTAATA	TCGACAATACGCTGCTTGAA	4	*Toxoplasma gondii*
AAAGCCATTTCCGTATGCTG	TGTACTCGCATTTGGCTGAC	3	*Yersinia spp*
AGAGGCAGGCAGACTTCAAA	CCAGCAAACACCCATATTGA	N/A	Seoul hantavirus
CGAGAAAGACTGGAATGAGTGA	CACGCGTTGAAAGCATGTA	N/A	Puumala hantavirus
TGCTGGCTTGAAAAGAATCAA	TCCTGAATAAAGCGAGGTCA	N/A	Hantavirus spp
ATGACACGATTGCCAATACTTC	CTTACTGTAGTGTATGAGACAGC	3	Cowpox virus
ACGCGGATCCTTCTACTCCT	GGCTGATCGAATCTTTCCAA	2	*Leptospira interogans*
GCTCTTGCAGCTTCTATGTT	CCGCCAACCTGACGGGCAATGG	N/A	*Richettsia typhi*
CCTGGGAAAACCACTGCACA	TTGACAAGGTGCAGGAAGATGC	5	Lymphocytic choriomeningitis virus (LCMV)
TGTGAGCCAGTCGTGATTGT	AAGCCTCTTGCAATTCCTCA	5	Encephalomyocarditis virus (EMCV)
ACAGCCTTCGAAGTGTTGAA	TGGGCGACAACTCAACATG	5	EEHV1
AGTGCATCGTCGGAATATGC	TCCCTGAGAGCGGCTTTT	5	EEHV1

* A number of probes were confirmed to hybridise in a previous study by Giles et al (Giles et al., 2015) and are as stated in the study, these are highlighted in red text. Those highlighted in blue were also from this previous study, though positive control material was not available, so their hybridisation status was not confirmed. Those highlighted in purple text were developed for this study; positive control material was available for both EEHV1 and EMCV to confirm the probes hybridised.

7.7.6.1 Additional probes

All probes were designed using Primer3 software and manufactured by Eurofins, Germany. Each probe was designed to be 60bp long. EEHV probes were designed using the Elephantid herpesvirus 1 Raman strain sequence (KC462165.1) obtained from the NCBI nucleotide database. EEHV1_UPS_1-5 probe sequences were designed for the EE10 membrane protein gene of EEHV and EEHV1_UPS_11-15, were designed for the EE34 membrane protein gene (Table 29). All EEHV probes were designed to detect EEHV1 only. EMCV probes EMCV_UPS_1-5 were designed using the Encephalomyocarditis virus strain ATCC VR-129B complete genome sequence (KM269482.1) also on the NCBI database, the genome is not annotated this means gene information is not currently available (Table 30). All EMCV probes were designed to detect multiple strains of EMCV.

Table 30: Additional EEHV and EMCV probes designed for the microarray

Forward primer sequence (5" to 3" sequence)	Reverse primer sequence (5" to 3" sequence)	Probe name	Probes (5" to 3" sequence)	Start position	End position	Target pathogen
ACAGCCTTCGAAGTGTTGAA	TGGGCGACAACTCAACATG	EEHV1_UPS_1	TTGGCTAGGTGTACGGCTCGTGACGTAGGCAGATAAATAGAGATGATTTGGGGCGGTTCT	106129	106188	EEHV1
		EEHV1_UPS_2	CTGACTGTGTATCCCACCCAACAAGAATCTGCTATCGTTAGCTTTCACCACCGTACTGAT	106437	106496	EEHV1
		EEHV1_UPS_3	GCTCGTAATAGTCGTCTGTCTCATCGATTCCCCTCTTTGTAAGACTAACAGGGAAGTTCC	106058	106117	EEHV1
		EEHV1_UPS_4	ACGCCGGCAATGCAGAAACGACGTTAGTTGTAGTGTTTGTTGCAGTAACTGCGTGTCCGA	105926	105985	EEHV1
		EEHV1_UPS_5	AAATTATTCTGGCAGACGCACTATTGGCAGCCGTTTCCGAATCATCCATACAACCATCTG	106379	106435	EEHV1
AGTGCATCGTCGGAATATGC	TCCCTGAGAGCGGCTTTT	EEHV1_UPS_11	ACCGCAGTTAATCATAGCGCTAGAGTACTGCCTTCTGATTCACAGACTACAGGACCTGCA	148004	148063	EEHV1
		EEHV1_UPS_12	TACGCTTTCAAGAAATGGCGACACACGTTGAACCTAGGTCTGATCGGAAACGACATATTC	147738	147797	EEHV1
		EEHV1_UPS_13	CTCTTACTAGCCGCGACTCTCATCTCTCTAAAGTTGACAGAATTTCCAAAAAGCCGCTCT	148125	148184	EEHV1
		EEHV1_UPS_14	CTGGGAACGGTCATAGCCATGTTTGTCCTCGGCATGATAGTGAAAAGGTTCAGTAACCAG	147801	147860	EEHV1
		EEHV1_UPS_15	AGATGCTAACATAACGCAAGAGGTAGAGAATCGCTTCTACATGGAATATCTGGACTGTC	147628	147687	EEHV1
TGTGAGCCAGTCGTGATTGT	AAGCCTCTTGCAATTCCTCA	EMCV_UPS_1	CGCGGGGCAAGGGAAATCTTTATCAAGTCAGGTTATTGCCCAGGCCGTCTCCAAGACCAT	4788	4847	EMCV
		EMCV_UPS_2	CCGGGTATAAGGTTTTGGATGTTGAAAGAGCCTTTAGGCCTACCGGTGAGGCTCCTCTTC	5180	5239	EMCV
		EMCV_UPS_3	AGACGAGGTCAGTTTCCATTCCGTAGTCCAGCAGCTTAAAGCAAGACAGGAAGCGACAAGA	5412	5469	EMCV
		EMCV_UPS_4	TTTGCAGCAATAATGGATGATCTAGGGCAAAATCCTGATGGCTCTGATTTCACTACGTTC	4915	4974	EMCV
		EMCV_UPS_5	TGTGGTGGCAACTACCAATCTGCCTGAGTTTAGACCTGTCACAATAGCCCATTACCCTGC	5055	5114	EMCV

7.7.6.2 Real Time PCR (RT-PCR)

RNA was extracted as previously described, reverse transcription (RT) was performed to allow amplification of RNA viruses by PCR; enabling the formation of cDNA. RT-PCR was performed as described by Giles (2015). The following components were added to a total volume of 13µl in a PCR tube: 1µl random hexamer primer (40uM), 1µl dNTP mix, 10µl water and 1µl template (minimum of 50ng). In a thermal cycler the samples were heated to 65°C for 5 minutes, and then kept on ice for at least 1 minute. A total of 7µl of superscript III mix (Invitrogen, USA) (4µl 5x reverse transcriptase reaction buffer, 1µl 0.1M DTT, 1µl RNase inhibitor, and 1µl superscript III reverse transcriptase) was then added to each reaction. The samples were then incubated at 25°C for 5 minutes followed by one hour at 50°C, a final incubation of 15 minutes at 70°C was performed to ensure the RT enzyme was inactivated.

7.7.6.3 Multiplex PCR

7.7.6.3.1 Primers and probes

Primers and probes developed for use in a microarray assay must be highly specific for the target pathogen. There are several factors that need to be considered and must be

balanced in the probe design process including; sensitivity, specificity, noise and bias (Hukkanen et al., 1999).

For this study, a multiplex PCR was developed to amplify a number of pathogens so they could then be detected in using the microarray including EEHV1, EMCV, *Leptospira interrogans*, Lymphocytic choriomeningitis, cowpox virus, *M. bovis*, *M. tuberculosis*, *M. avium subspecies paratuberculosis*, *Rickettsia typhi*, Puumala hantavirus, Seoul hantavirus, *Yersinia spp* and *T.gondii* using a series of primers.

A 10x primer mix was made containing 2mM of each primer. The cycling conditions for this reaction can be seen in Table 31. Denaturation, annealing and extension steps were repeated for 40 cycles.

Table 31: Cycling conditions for multiplex PCR.

Step	Temperature (°C)	Time
Initial denaturation	94	2 min
Denaturation	94	30 sec
Annealing	10	1 min
Extension	72	1 min
Final extension	72	10 min
Final hold	4	indefinitely

7.7.6.4 Biotin labelling and PCR

PCR products obtained from multiplex PCR (described previously) were labelled with biotin prior to hybridisation. The protocol followed was performed as described by Giles (2015).

The reaction volume was 50µl, consisting of 30 µl nuclease free water, 3µl 25mM $MgCl^2$ (Invitrogen, USA), 5µl 10X Mg free buffer (Invitrogen, USA), 5µl 10x biotin labelling mix (for a 100µl total: 1µl dATP, 1µl dGTP, 1µl dCTP, 0.65µl dTTP, 35µl Biotin-dUTP, 61.35µl water, 0.5µl 10x array primer mix), 0.5µl taq polymerase (Invitrogen, USA) and 5µl of PCR product. The PCR cycling conditions were as follows: 94°C for 10 sec, then 25 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 2 min, this was followed by a final extension of 2 min at 72°C.

7.7.6.5 Hybridisation

Hybridisation was conducted using a protocol for array strips as described by Giles et al. (2015); for this study array strips were replaced with an array tube (AT) platform.

7.7.7 Confirmatory EMCV testing by PCR

7.7.7.1 Reverse transcription of RNA

Each reaction consisted of 1µl random hexamer primer and 14µl RNA template, this was put into a thermal cycler (Techne TC-512 thermal cycler) for 10 min at 70°C, following this 5ul M-MLV buffer (Invitrogen, USA), 1.25µl dNTPS (New England Biolabs, USA), 1µl M-MLV enzyme (Invitrogen, USA) and 3µl

RNase/DNase free H_2O was added to each tube and incubated at 37°C for one hour.

7.7.7.2 Primers for cDNA and EMCV detection

Two sets of primers were used: one to detect IFNγ (to confirm the presence of Asian elephant cDNA), and one to detect the presence of EMCV (Table 32), the primers used were synthesised by Eurofins, Germany. IFN sequences were obtained from a previous publication (Stanton et al., 2010). EMCV primers were developed using Primer3 software.

Table 32: Primers used to amplify IFNγ and EMCV. IFNγ primers were used to confirm the presence of Asian elephant cDNA.

Primer	Sequence (5"-3")
IFNγ forward	GCGTGAAGACCCTTGAGGAA
IFNγ reverse	TCATTTACCGGAGTTTGCATCA
EMCV forward	TGTGGCACAAGCTCCAGTAG
EMCV reverse	TGTTCATCTGTCGCTTCCTG

7.7.7.3 Cycling conditions

Both PCRs for the detection of cDNA and EMCV utilised the same cycling conditions, denaturation, annealing and extension steps were repeated for 40 cycles (Table 33).

Table 33: Cycling conditions for the detection of cDNA and EMCV.

Step	Temperature (°C)	Time
Initial denaturation	95	2 min
Denaturation	95	2 min
Annealing	60	30 sec
Extension	72	1 min
Final extension	72	5 min
Final hold	4	indefinitely

7.7.7.4 Visualisation

All PCR products were visualised using gel electrophoresis on a 3% agarose gel using Nancy 520 fluorescent stain.

7.8 Results

On the microarray, the majority of heart samples from EEHV-positive animals tested positive for the presence of EEHV (3/4), compared to 3/5 of liver samples, 1/4 of kidney samples, and no lung samples were positive.

Iconoclust software used for analysing the array chip after hybridisation normalises the signal, this is after an algorithm determines the intensity of the spot and the local background noise. Signals range from 0 to 1, with 0 being negative and 1 being the highest possible signal. Normalisation is the local background noise subtracted from the average intensity of the spot.

For both heart and liver samples which tested positive, positive hybridisation signal intensity was defined as those at 0.2 or above, between 0.15 and 0.2 were classed as weak positives or further investigation needed. Heart and liver tissue from UON_1329 tested positive for EEHV only. Biotin markers were located in the top right
and bottom left corners of the array tube, these spots were used as positive controls. All spots that were presented with a signal are probes for EEHV1, no co-pathogens were present in any samples obtained from UON_1329 (Figure 43).

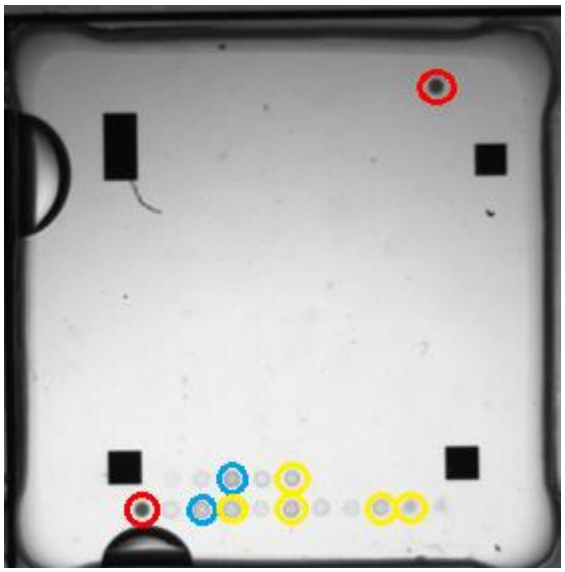


Figure 43: Hybridisation signals on the AT from heart tissue from UON_1329. The image shows a number of hybridisation spots of varying strengths. Two biotin markers can be visualised in the top right hand and bottom left hand corner and are highlighted with red rings. Positive hybridisation signals for EEHV1 are highlighted by blue rings and those that were classed as weak positives are highlighted with yellow rings.

In contrast, the liver from this animal presented with 4 positive hybridisation signals and 3 weaker signals; no signals were present for co-pathogens (Table 34). Samples from both

the lung and kidney samples from UON_1329 were negative for all pathogens. The cut off point for positive signals was 0.2, and those between 0.15 and 0.2 were classed as weak positives or requiring further investigation

Table 34: Array signal results for UON_1329 showing the presence of EEHV1 DNA in both the heart and liver samples. There were no positive or weak positive signals in the lung of kidney.

UON_1329		
Probe	**Tissue**	
	Heart	**Liver**
EEHV_UPS_2	0.21	0.22
EEHV_UPS_3	0.17	0.18
EEHV_UPS_5	0.19	0.19
EEHV_UPS_8	0.15	0.21
EEHV_UPS_9	0.17	0.29
EEHV_UPS_13	0.24	0.28
EEHV_UPS_15	0.18	0.19

EEHV was also detected in the heart and liver obtained from UON_1189, 11 positive signals and 1 weaker signal for the heart and 10 positive signals and 3 weaker for the liver (Table 35), cut off points were used as described previously. A greater number of probes hybridized in comparison to UON_1329. DNA/RNA extracted from a kidney sample, showed positive results for *Hantavirus spp* and a weaker

positive result for cowpox virus; confirmatory PCR was performed as further investigation was needed to confirm the presence of these pathogens. Probes with no positive hybridisation signal are show as N/A in Table 35.

Table 35: Array signal results for UON_1189 showing the presence of EEHV1 DNA in both the heart and liver samples, and *Hantavirus spp* and cowpox virus in the kidney.

UON_1189			
Probe	**Tissue**		
	Heart	**Liver**	**Kidney**
EEHV_UPS_1	N/A	0.17	N/A
EEHV_UPS_2	0.33	0.32	N/A
EEHV_UPS_3	0.21	0.20	N/A
EEHV_UPS_4	0.65	N/A	N/A
EEHV_UPS_5	0.33	0.31	N/A
EEHV_UPS_6	0.24	0.25	N/A
EEHV_UPS_7	0.17	0.19	N/A
EEHV_UPS_8	0.42	0.43	N/A
EEHV_UPS_9	0.44	0.50	N/A
EEHV_UPS_10	0.27	0.24	N/A
EEHV_UPS_11	N/A	N/A	N/A
EEHV_UPS_12	N/A	0.17	N/A
EEHV_UPS_13	0.44	0.44	N/A
EEHV_UPS_14	0.23	0.20	N/A
EEHV_UPS_15	0.34	0.37	N/A
HANTA_L833	N/A	N/A	0.20
Cowpox virus	N/A	N/A	0.17

Heart and liver tissues from UON_1149 were positive for EEHV. The liver sample presented with the most hybridised probes with three positives. By contrast, for the heart sample

there was one positive signal and one weak positive. The kidney sample had only one weak positive hybridised probe, this was the only EEHV positive kidney sample during the study (Table 36), cut off point used as described previously.

Table 36: Array signal results for UON_1149 showing the presence of EEHV1 DNA in the heart, liver and kidney.

UON_1149			
Probe	**Tissue**		
	Heart	**Liver**	**Kidney**
EEHV1_UPS_13	0.25	0.32	0.19
EEHV1_UPS_14	N/A	0.20	N/A
EEHV1_UPS_15	0.17	0.24	N/A

The array signal results were compared to qPCR data; this can be compared with the hybridisation signal from the array to determine if the results are semi-quantitative. Based on the amount of EEHV present in samples from UON_1189 (as determined by qPCR), the highest load was in the heart followed by liver, lung and the lowest in the kidney. On the array, the signal was the highest for the heart and liver, whereas there were no positive signals for the kidney and lung samples. EEHV was also detected by qPCR in the samples obtained from UON_1329, the highest amount of EEHV was present in the liver and the heart, and barely detectable

amounts were present in the kidney and lung samples. The array method similarly detected the highest EEHV load in the liver and heart, while no positive signals were obtained for the kidney and lung samples (Figure 44), this would suggest that the microarray results are semi-quantitative.

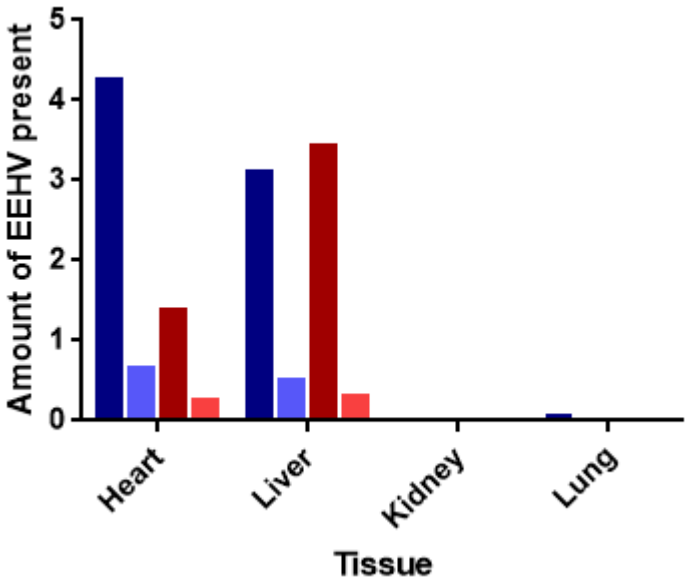


Figure 44: A comparison of EEHV virus DNA load using qPCR and microarray. The amount of EEHV using the qPCR method was calculated by normalising against IFNγ, this is compared to the signal value obtained from the array method**.** Results from UON_1189 are in dark blue (qPCR) and light blue (array), and those from UON_1329 are in dark red (qPCR) and light red (array).

Statistics was performed on the positive hybridisation signals from the heart and liver samples obtained from UON_1189 using the Pearson product-moment correlation coefficient. This suggests that there is a strong positive correlation between the amount of EEHV present in samples using PCR and using the microarray (r=0.994) (Figure 45). The results also show a high significance (p=0.0006).

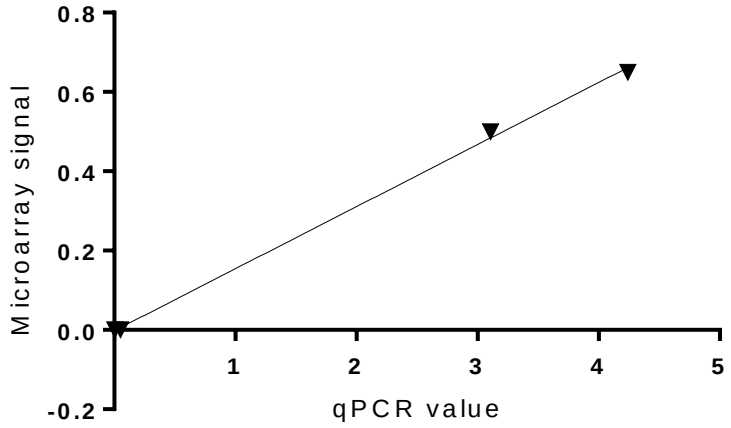


Figure 45: A correlation between qPCR normalised value and microarray hybridisation signal for UON_1189. The correlation between the two values indicates that a higher microarray signal correlated with a higher virus DNA load as measured by qPCR. The results show a positive correlation with a Pearson product-moment correlation coefficient of 0.994 and is statistically significant with a p value of 0.0006. The equation of the line is Y = 0.1565*X - 0.002169.

Statistics performed on the hybridisation signal data (heart and liver) from UON_1329 using the Pearson product-moment correlation coefficient, suggests that there is a strong positive correlation between the values (r=0.9174) (Figure 46).

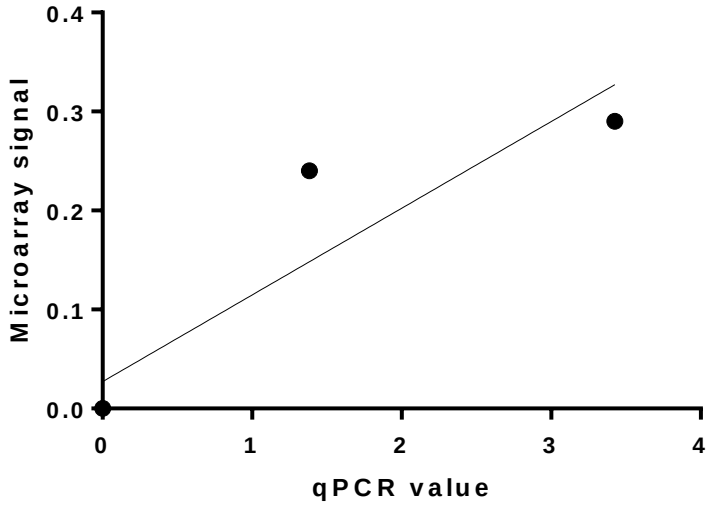


Figure 46: A correlation between qPCR normalised value and microarray hybridisation signal for UON_1329. The correlation between the two values suggests that a higher microarray signal correlated with a higher virus DNA load as measured by qPCR. The results show a positive correlation with a Pearson coefficient of 0.917, although it was not statistically significant, with a p value of 0.0826. The equation of the line is Y = 0.08755*X + 0.02710.

Trunk washes obtained from herds in the UK and in Switzerland were also investigated. Trunk washes from four animals at a zoological collection in the UK were negative for all pathogens, whereas of the samples obtained from animals in Switzerland, 2/5 samples were positive for *Mycobacterium spp*, another was determined to be weak positive for *Mycobacterium spp*, and 2/5 were classed as negative for all pathogens tested (Table 37), cut off points as described previously.

Table 37: Array signal results for five animals from a Swiss collection showing the presence of *Mycobacterium* DNA in three samples.

	UON_1140	**UON_1625**	**UON_1089**	**UON_1093**	**UON_1175**
Probe					
Mycobacterium_02	N/A	0.05	0.11	0.13	0.14
Mycobacterium_03	N/A	0.09	0.20	0.07	0.25

7.8.1 EMCV PCR

Available tissue samples were further screened for the presence of EMCV by PCR using specific primers. Heart, liver, lung and kidney samples from UON_1189 and UON_1329, and heart, liver and kidney tissue from UON_1149, all tested negative for the presence of EMCV (Figure 47).

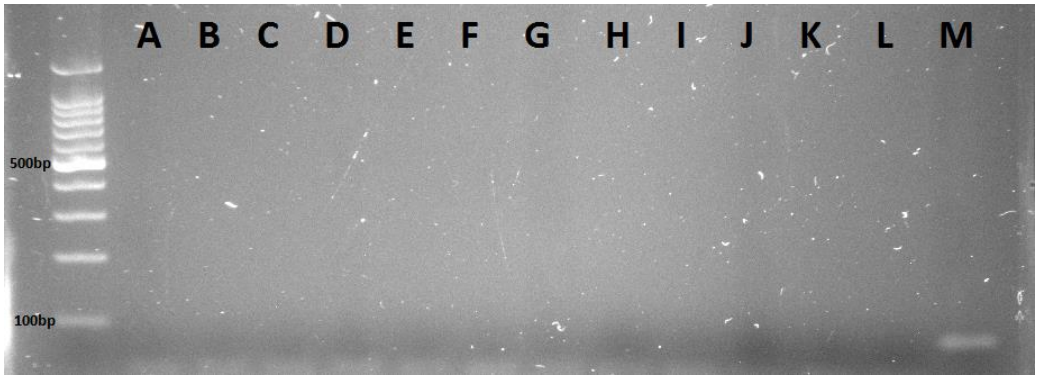


Figure 47: Gel electrophoresis using a 3% agarose gel suggesting all samples were negative for the presence of EMCV using specific primers. Lanes A-K are elephants tissue samples, A-D UON_1189 heart, liver, lung and kidney, E-H UON_1329 heart, liver lung and kidney, and I-K UON_1149 heart, liver and kidney. Lanes L and M are negative and positive control, L is the negative control and M is the positive control (EMCV cDNA) (84bp).

The tissue samples used for EMCV detection by PCR were also screened to determine the presence of cDNA. This was performed to confirm samples were true negatives, and that EMCV was not present due to the lack of cDNA in the samples, or presence of PCR inhibitors. Heart, liver and kidney samples from UON_1189 contained cDNA; however, the lung sample did not. All samples obtained from UON_1329 contained cDNA (Figure 48). The positive control material was EMCV (Cell culture adapted) ATCC VR-129 (M-variant) isolated from virus grown in cell culture.

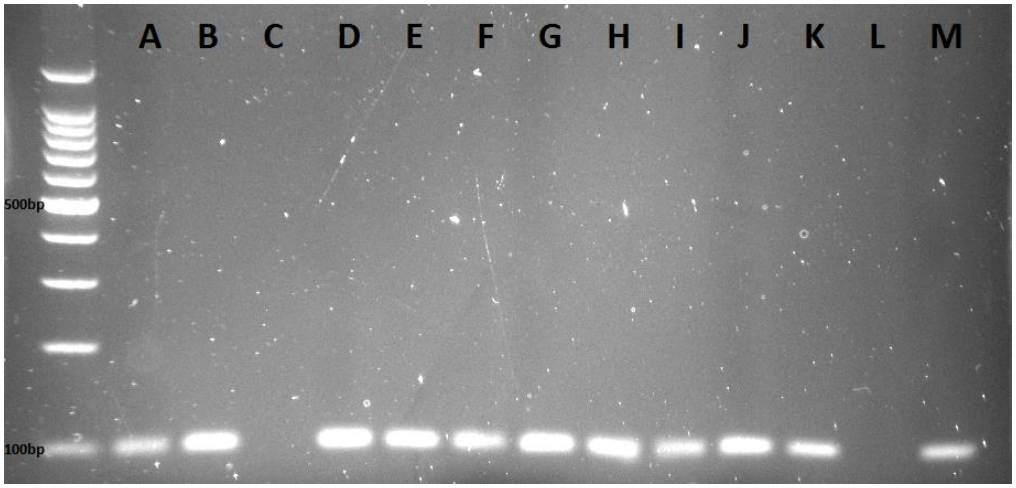


Figure 48: Gel electrophoresis using a 3% agarose gel showing all but one sample contained elephant cDNA. Lanes A-D: UON_1189 heart, liver, lung and kidney, Lanes E-H: UON_1329 heart, liver lung and kidney, and Lanes I-K: UON_1149 heart, liver and kidney. Lane L was the negative control and Lane M was the positive control, with the expected bp size of 100bp.

As the number of viral copies was unknown, PCR was performed on a serial dilution of the EMCV positive control material, the starting concentration of which was not available. EMCV cDNA could be detected at the lowest concentration tested (1 in 100,000) (Figure 49).

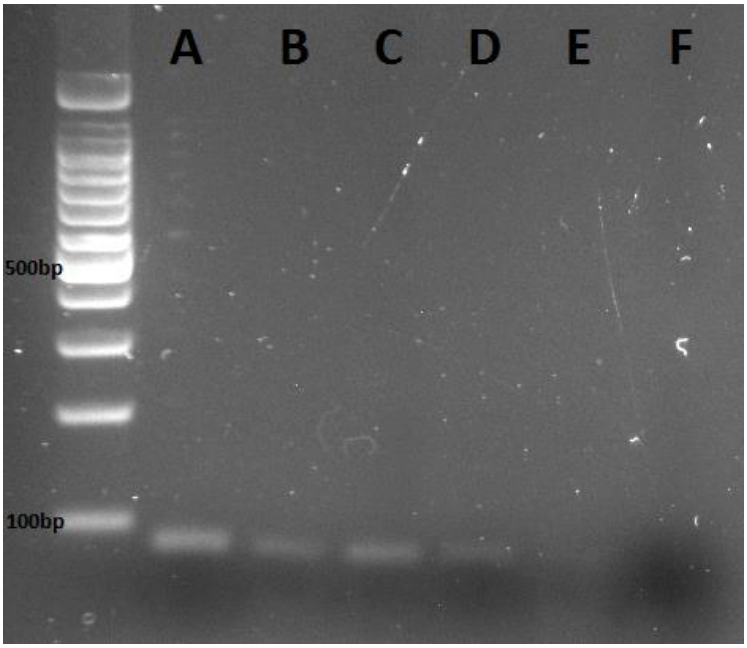


Figure 49: Gel electrophoresis using a 3% agarose gel showing pure EMCV cDNA and a serial dilution. Lane A = pure EMCV cDNA derived from culture, Lane B = 1/10, Lane C = 1/100, Lane D = 1/1000, Lane E = 1/10000 and Lane F = negative control.

7.9 Discussion

This study investigated the potential presence of co-pathogens in tissue samples from EEHV1 infected Asian elephants. It is currently unknown if co-pathogens play a role in the development of haemorrhagic disease, which is currently attributed to EEHV infection. There was evidence of co-pathogens (Hantavirus and cowpox virus) present in the tissue sample from UON_1189, however, further testing showed this to be non-specific binding/cross hybridization. However, a number of trunk wash DNA samples tested positively for the presence of *Mycobacteria spp,* this was subsequently confirmed via PCR.

The cut off values used for determining positive and negative samples on microarrays have not been standardised and can be variable. The microarray assay cut off values were set at a low level of >0.2 for positives, 0.15-0.19 were classed as 'further investigation by PCR needed', and below 0.15 were classed as negative. A threshold value of 0.2 was selected as there was very little background noise, and signals were weak for positive control samples. A low signal in this case could be due to probes not performing effectively. By contrast, the use of a related version of this microarray for a study of rodent

pathogens, described by Giles et al. (2015), used a cut off threshold of >0.4.

Results confirmed that EEHV was present in 7/18 of the clinical case samples from the five elephants studied.

Four of these five animals were positive for EEHV; this had previously been determined by qPCR. Heart and liver samples appeared to give the strongest positive results, while one kidney sample gave a weak positive result for EEHV. The strength of the hybridisation signal reflected the quantitative results from the prior qPCR testing for both UON_1329 and UON_1189. However, while results from UON_1189 were clearly significant, those from UON_1329 were not, this may be due to the low number of data points obtained and analysed. Array results from heart and liver samples would suggest that the microarray method is potentially semi quantitative. However, EEHV was not detected by microarray in either the kidney or the lung samples, although it had been detected by qPCR in both samples at low levels. This suggests that the microarray is not sensitive enough to detect low levels of EEHV. This is the likely explain explanation as to why EEHV was not detected in trunk washes using the microarray, but has previously been detected using qPCR. Trunk washes are usually obtained from animals that are asymptomatic and

are sporadically shedding EEHV and are not showing clinical signs, lower levels of the virus are therefore expected. Cross hybridisation/non-specific binding was experienced during this study (as described above), it is a common problem when using microarrays, and is thought to be the main reason for diminished microarray result quality, once improved by either probe amendment or reviewing the hybridisation process, the validity of the results may increase (Koltai and Weingarten-Baror, 2008). However, there are a number of studies where cross hybridisation is the aim, Adjaye et al. (2004) successfully developed an array to detect both human and bovine orthologous genes.

To confirm array findings that samples tested using the array were negative for EMCV, a PCR was performed to check for EMCV; results confirmed the negative finding. By contrast, a previous study investigating the presence of co-pathogens in tissue samples from EEHV positive elephants identified one animal which presented with a concurrent infection of both EEHV and EMCV (Richman et al., 1999).

All trunk washes tested negative for EEHV on the microarrays; this was unexpected, as previous trunk wash samples for 8/9 of these animals had tested positive for EEHV using qPCR.

However, *Mycobacterium spp* was detected in the trunk washes of 3 animals from Switzerland, further testing by PCR was not able to confirm the species present, however, it was confirmed not to be *M.bovis*, *M.bovis* BCG, *M. paratuberculosis* or *M. microti.* This would suggest a non-pathogenic species of Mycobacteria was present in the sample; this would likely be an environmental Mycobacteria species. Looking for *Mycobacteria spp* in elephant oro-nasal secretions can present additional challenges, depending on the findings, as the presence of *M.bovis* or *M.tuberculosis* would require immediate destruction of the samples, and notification of the relevant authorities.

The array was not as sensitive as PCR or qPCR in the detection of low levels of EEHV, this was determined due to EEHV not being detected in trunk washes samples that had previously tested positive by qPCR. Previous studies have also reported poor microarray sensitivity, this may be due to poor hybridisation process or issues with reagents, such as Relógio et al. (2002) found higher concentrations (70-80%) of formamide resulted in very poor sensitivity. Probe design is also a key factor in regards to microarray sensitivity. Binder et al. (2004) reported that probes with an increased number of C nucleotides per oligomer showed an increased sensitivity, in

comparison to low sensitivity probes containing high numbers of G and T nucleotides, the study found probes with a high number of A nucleotides appear to be the least sensitive.

Further redesigns could include using longer probe sequences, which would allow the detection of related species. It has been suggested than longer probes up to 65-70bp are more effective, this could be due to more base pairs are available for hybridisation, however, if the probes are not correctly designed longer probes may result in more cross-hybridisation. Using longer probes would potentially produce a stronger signal (Shaw et al., 1987, Hukkanen et al., 1999). However, for this study shorter probe sequences (15-30bp) may be more useful, as this would allow for greater specificity and would allow strain identification (Wang et al., 2003). Despite this, the number of strains identified may be limited, this may not be beneficial for viruses like EMCV where there are a large number of strains, designing probes within short sequences and covering a large number of strains may be difficult. Further adjustments to improve the microarray could also be made. This study uses sodium chloride and sodium citrate (SSC) and formamide and dithiothreitol (DTT) in the hybridisation buffer whereas the addition of other components may increase the efficiency of the microarray. The addition of

dextran sulfate to the hybridisation solution may increase the rate of hybridisation resulting in a stronger signal (Ku et al., 2004). This study added Denhardt's solution into the hybridisation buffer to reduce the chance of nonspecific binding. However, other components such as sonicated salmon sperm DNA and poly A can also be used (Russell et al., 2008). Changes could also be made to the hybridisation temperature, using the correct temperature is important during this step, as a temperature that is too low will cause a decrease in specificity due to an increase in non-specific binding. In contrast if the temperature is too high sensitivity will decrease due to a reduction in the hybridisation of the specific target (Russell et al., 2008).

This study also only used tissues from 5 dead animals and 9 asymptomatic animals; to make this study more statistically robust, more samples from a larger number of animals would be required. The availability of tissues for this type of assay is also challenging; tissue from clinical cases is difficult to obtain as the necropsy of zoo elephants may not occur until several days after death. This makes collecting tissue samples for RNA extraction difficult, as tissue must be placed in RNA later as soon as possible after death as to prevent degradation. This likely delay in sample collection, and the resulting probable

degradation of RNA, may have contributed to lack of detection of any RNA pathogens by the microarrays.

We found no evidence for a role for EMCV or other possible co-pathogens in some clinical cases of EEHV. Our findings suggest it would be beneficial to investigate clinical cases of EEHV for multiple pathogens using a more sensitive assay, such as PCR or qPCR. Further studies are needed to confirm the quantitative relationship between microarray signals and qPCR values. Investigating pathogens present in rodent populations in captive elephant environments may also help to detect an outbreak of diseases such as anthrax, cowpox virus, leptospirosis, plague, toxoplasmosis, and EMCV (discussed in 1.4.2) which have all been reported in elephants, and are thought to be transmitted by infected rodents to elephants. If an infectious pathogen is detected in the local rodent population and an outbreak is suspected steps can be taken to reduce the potential of transmission, this may include pest control.

Chapter 8 – Main conclusions

8 General discussion

The main objectives of this thesis included investigating a number of aspects of EEHV; 1. Is there a link between EEHV shedding and the physiological stress of pregnancy? 2. The role of host genetics and potential co-pathogens. 3. The culture of elephant umbilical endothelial cells as a potential tool for virus isolation 4. A comparison of the virus DNA loads in the tissues of two dead animals that died with clinical signs consistent with EEHV. This thesis has added further information to that currently available for EEHV

In the study of EEHV there are lots of unknowns, including what stressors or triggers cause a reactivation of EEHV or increase EEHV shedding. Previous studies had suggested that the physiological stress of pregnancy may increase the amount or frequency the animal sheds EEHV in comparison to their non-pregnant counterparts (Stanton et al., 2010). This thesis has shown no evidence for increase in shedding associated with pregnancy; however, the main limitation of the current study is the limited number of animals available for inclusion. Further studies are warranted to confirm the findings, however, due to the small size of captive herds, the number of animals in each cohort is likely to be small, especially the number of pregnant animals. In order to gain

maximum value from each study and to identify potential stressors, thorough notes should be kept by keepers although this may not always be possible.

It is currently not known if anti-viral drugs are effective in treating clinical EEHV. One way to determine this would be to grow EEHV in culture and treat the culture with different drugs to determine their efficacy. This is currently not possible as a cell culture system for the growth of EEHV has not been developed. As discussed in chapter 4, cell culture work successfully isolated cells. However, a subsequent revival of cells from storage was not successful. In the future further cells could be isolated using this method; however, it may not be easy to achieve due to the difficulties in obtaining fresh Asian elephant umbilical cords, contamination issues, and the limited number of animals in the UK. Also in protected contact environments the umbilical cord first must be retrieved by members of staff, this may not always be possible. Once a cell culture line has been established, the cells can be made immortal which allows for experimentation without starting from a primary cell line. Immortality can be established in a number of ways, including introducing telomerase and telomerase reverse transcriptase (TERT) into the cells, which increases stability making them immortal, over expressing

hTERT has also show to immortalise endothelial cell (Chang et al., 2005, Tsai et al., 2010). Cells can also be immortalised by the introduction of a viral vector such as SV40 or human papillomavirus (Jha et al., 1998, Hubbard and Ozer, 1999). The main advantage of cell immortality is that there is indefinite proliferation which allows for prolonged periods of study. However, the main disadvantage is that once cells become immortalised they may behave differently in comparison to their origin (Burdall et al., 2003).

This thesis presents a genogram showing how Asian elephants in Europe and the USA are related, and highlights those whose deaths are thought to be associated with EEHV. The genogram highlights that there may be a potential genetic or familial component linking EEHV associated deaths. Whilst a genetic link is mechanistically possible as it has been reported that HHV6 integrates into the telomeres of chromosomes and can be inherited (Daibata et al., 1999), it would need further study to investigate this further in the case of EEHV. A Fluorescent In Situ Hybridization (FISH) metaphase chromosome spread could be used, this method was used by Tanaka-Taya et al. (2004) to determine if HHV6 had been inherited in an integrated form. If future work confirms a link between genetics and EEHV this may create potential issues as it may

force certain animals to be removed from breeding programmes or to be re-located causing issues for many establishments. It would also be difficult to increase genetic diversity in captive environments as it would not be ethical to take new animals from the wild to increase the genetic pool. However, wild animals contributing to the captive genetic pool by the use of cryopreservation has been a long standing idea (Ballou, 1992). This type of genetic enrichment using frozen and thawed semen as a form of genetic enrichment has been performed successfully in the insemination of a captive African elephant in Austria (Hildebrandt et al., 2012). As artificial insemination has been successful in the production of Asian elephant calves, this method may also be successful in expanding the captive Asian elephant gene pool, however, further research is needed (Hildebrandt et al., 2012, Hermes et al., 2013).

Further investigation is needed into the potential involvement of co-pathogens in EEHV associated deaths, this thesis found none of the pathogens selected were detected in EEHV positive tissue samples. This research has provided evidence to suggest microarrays may not be the most appropriate way of screening for multiple pathogens in the case of EEHV associated deaths. Future work on co-pathogens is possible;

however, the relationship between co-pathogens and EEHV is still unclear. To establish whether a co-pathogen is involved in EEHV associated deaths would be difficult, as it would require a large number of samples from a large number of deaths associated with EEHV. This is a major limiting factor when studying EEHV as there is limited number and availability of samples; it is therefore difficult for studies to be statistically significant. When investigating multiple pathogens, it can difficult, due to a number of factors including the nature of some pathogens, such as if TB was found to be present this may cause implications for laboratory containment and for the establishment keeping the positive animal.

However, there are other methods that could be used to look at multiple pathogens in EEHV related deaths such as next generation sequencing and multiplex PCR. Next generation sequencing has been used to screen samples to determine the multiple pathogens present, the identification of viral strains and the identification of unknown viruses (Hummelen et al., 2010, Beerenwinkel and Zagordi, 2011, Yozwiak et al., 2012). This technique may be useful in the study of EEHV in the identification of potential co-pathogens; however, it would need further investigation to determine the relationship between co-pathogens.

EEHV appears to be a problem in both captive and wild populations, however, it is currently unknown if studying EEHV in captive elephants can be translated into wild populations. Deaths from EEHV may be seen more frequently in captive populations due to the elimination of other threats such as poaching and habitat loss. There are many arguments against keeping elephants in captive environments such as implications on their welfare, differences in social groupings, lack of space, early removal from mother and isolation (Clubb and Mason, 2002, Rees, 2009). Keeping elephants in zoos can be difficult, especially keeping bulls due to their aggression; as such this has resulted in a number of female only herds (Cooper et al., 1990, Frei, 2016). A zoo in the UK recently announced the relocation of their all-female Asian elephant herd due to the reproductive potential of two females; they report that breeding is better when a male is present in comparison to artificial insemination, and that the move will help with the long term survival of the species (Twycross Zoo, 2017).

The literature suggests that many zoos are involved in elephant conservation programmes in range countries; this would suggest that zoos are having a positive impact by providing funds for conservation. However, if the end result in

studying EEHV is to conserve wild elephants, funding may be better spent on issues having a greater impact on wild elephants such as human elephant conflict, poaching and habitat loss.

Chapter 9 – Future work

9 Future work

Future work arising from this study may include a further longitudinal study, investigating other potential stressors and establishing their impact on the elephants' susceptibility to EEHV. This would need the inclusion of appropriate glucocorticoid measurements to determine stress levels. However, careful interpretation would be needed as glucocorticoid measurements are often used an indicator of stress, but they can increase under a number of circumstances such as increased activity levels, arousal or excitement. Other potential stressors/stress relievers that could be investigated to determine their impact on the elephants' susceptibility could include the transfer of animals to another collection, death of a herd mate, changes made to nutrition, enrichment provision, and the introduction of new animals, sickness due to other illnesses, public interaction and changes to enclosures.

As we have successfully developed a method for culturing endothelial cells, this could lead to further attempts in establishing stocks of endothelial cells for use in attempts to create stocks of EEHV for infection, which could be used in drug efficacy and vaccine development studies. An EEHV infection could then be attempted, to determine if cell to cell

transmission occurs. A sample of infected tissue could be placed on to cultured cells, the supernatant could then be tested using PCR to determine if an infection was successful by measuring the amount of EEHV present. Establishing a cell culture system and the ability to grow EEHV in a laboratory environment would facilitate vaccine development and drug efficacy testing. Being able to establish stocks of the virus would allow for research without having samples from infected elephants. However, vaccine development is expensive, and it would be difficult to test in Asian elephants, due to the number of elephants that would be needed and also for ethical reasons. It may also be possible to establish an immortal cell line using hTERT vectors, this would allow cells to proliferate indefinitely and would avoid the issues encountered when using primary cell lines.

To determine the type of tissue to be used in an infection, it is important to establish which tissues have the highest virus DNA loads. There are currently a limited number of publications investigating the tissue tropism of EEHV1 by determining virus DNA load. The study determined EEHV1 to have a broad tissue tropism, with higher virus DNA loads particularly in the heart and the liver. Only two animals were included in this study; determining the tissue tropism of

EEHV1 in other deaths may provide further information on which tissues present with the highest virus DNA loads. Understanding tissue tropism is important for a number of reasons, such as it can impact on treatment, it can be useful in determining a proxy animal model and useful in comparing different strains (Cullen, no date). As the preliminary study within this thesis suggests the possibility of a genetic or familial link in susceptibility to the virus, future work could build on this. This could include investigating whether EEHV integrates into the chromosomes of Asian elephants; a fluorescence in situ hybridisation (FISH) test could be used to detect EEHV DNA on chromosomes. If EEHV DNA was present on chromosomes it could indicate the possibility of EEHV transmission having a heritability factor.

The genogram created within this study could be expanded or another genogram could be created which assesses all captive elated Asian elephants in western zoos. However, there may be limitations to this as the relatedness for each animal may be unknown or that only the mother is known.

We determined there were no pathogens from the selected panel present in EEHV positive tissues from four deaths. This study could also be built upon. The microarray was able to

detect EEHV and was comparable to qPCR data, suggesting it was semi-quantitative. A microarray would need to be validated for use in EEHV diagnostics; it may be a useful tool if all strains of EEHV could be detected in a sample. It may also be useful in determining if other pathogens are also present. For a microarray to be successful in determining pathogens within elephant samples the method would also need to be validated. Future work could also include screening further samples, and including other pathogens on the chip. The microarray performed poorly in the study outlined in this thesis, it would need a significant amount of development to enable it to be useful in diagnostics and screening. A PCR based assay for multiple gene targets could be used as alternative, a number of publications have shown that multiplex PCR is successful in determining a number of different pathogens or strains (Stockton et al., 1998, Oliveira and de Lencastre, 2002, Waggoner et al., 2016, Pham et al., 2017).

There are a number of challenges when working with endangered species. For example, importing and exporting samples can be difficult to obtain if the animal is listed under CITES, due to the need for import and export permits which limits the samples that can be obtained especially if there is a

time factor. There are also difficulties in obtaining samples from animals particularly if they are considered dangerous, as it may be required for the keeper to physically enter the animals' enclosure such as in the acquisition of a placenta. Working with endangered species may also mean certain experiments are limited to working with animal models such as drug trials and vaccine development, this is due to ethical issues and the number of animals available.

When looking at the challenges involved in working with endangered species it highlights an important question of whether Asian elephants should be kept in captivity. There is little information to suggest that keeping and breeding Asian elephants in zoos is justified, a number of breeding programmes have been developed to try to enable captive populations to become self-sustainable. However, there has been limited breeding success in zoo elephants leading to breeding programme failure (Taylor and Poole, 1998). It has been suggested that the breeding performance of captive Asian elephants located in logging camps is more comparable to wild elephants and better than those kept in zoos (Krishnamurthy and Wemmer, 1995, Taylor and Poole, 1998). There are also a number of welfare issues in relation to keeping elephants in captivity, such as poor healthy (obesity

and foot problems) and poor longevity. Keeping elephants in zoos may not currently contribute to wild Asian elephant conservation; to enable animals to be released into the wild zoos would need to be able to not only sustain the number of captive elephants but exceed them, a surplus would need to be achieved. To highlight the downward trend of elephant populations in captivity, using a population model to predict the population size of Asian elephants in North American zoos, Wiese (2000) suggested that the population will drop to around 10 individuals by 2050. Limited information is available for Asian elephant captive populations. In addition, there may be evidence to suggest that viruses such as EEHV and TB may have increased in captive populations due to the movement of animals between zoos, which would be counterproductive in the conservation effort (Richman et al., 1999, Mikota et al., 2000). Although there has been a significant monetary input into EEHV research from both public and academic funds, this may not be justified from a conservation standpoint, as currently there is limited information about how EEHV impacts wild populations, and it is unknown if studying EEHV in captive populations is going to be of benefit to wild populations. There is currently no evidence to suggest that addressing EEHV in captive populations will contribute to creating a sustainable captive population or conserving wild Asian elephants. Given

the challenges in studying EEHV and the development of potential vaccines or drug treatments, a greater impact on the survival of Asian elephants funding might be better directed into reducing habitat loss.

Chapter 10 - References

10 References

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Chapter 11 - Appendix

11 Appendix

11.1 Genogram

A genogram was constructed for 621 Asian elephants located in North America and Europe. Please see the supplemental CD disc for the complete genogram.