PROCEEDINGS

of the

Eleventh International Elephant Endotheliotropic Herpesvirus (EEHV) Workshop

15-17 May 2017

ZSL London Zoo and ZSL Whipsnade Zoo

Partially funded by the Zoological Society of London,
the Animal and Plant Health Agency and the International Elephant Foundation

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* Indicates 25 min talks, other talks are for 15 mins (each talk followed by 5 minutes question time)

Logistics

Monday, 15th of May		
10:00-18:00	Zoo visit	ZSL London Zoo All day access
18:00-21:00	Ice breaker	Prince Albert Suite ZSL London Zoo

Tuesday, 16th of May		
08:00-08:30	Coffee, tea and pastries	Huxley Theatre ZSL London Zoo
08:30-17:45	Presentations	Huxley Theatre ZSL London Zoo Coffee break between sessions Lunch break 12:30-13:30
18:00	Evening meal	Potential to join other delegates for visit to Camden Town (Pizzeria booked that seats 50 people) *Please register interest at registration desk**

Wednesday, 17th of May		
08:00-08:30	Coffee, tea and pastries	Huxley Theatre ZSL London Zoo
08:30-10:00	Presentations	Huxley Theatre ZSL London Zoo
10:00-10:30	Coffee break	Grab a drink to take with you on the bus by the entrance to the lecture theatre The bus to Whipsnade will leave promptly at 10:30
12:00-13:30	Practical elephant demonstration	Whipsnade Centre for Elephant Care
13:30-14:30	Lunch	Griffin Room ZSL Whipsnade Zoo
14:30-16:00	Elephant walk	Gather at Centre for Elephant Care before 14:30 promptly
14:30-15:00	Cross matching in practice	Veterinary Department – limited places (20) *Please register interest at registration desk***
15:00-16:00	Elephant haematology practical	Veterinary Department – limited places (9) *Please register interest at registration desk***

*) First come, first serve – undergraduate students excluded
**) Priority given to clinical veterinarians, nurses and technicians

Session 1: Icebreaker and introduction to the workshop

Opening of the 11th International EEHV Workshop – Welcome!

IMPACT OF ELEPHANT ENDOTHELIOTROPIC HERPESVIRUSES (EEHV)

Willem Schaftenaar

Rotterdam Zoo

Contact: w.schaftenaar@rotterdamzoo.nl

Session 2: EEHV across the globe and viral characteristics

ELEPHANT ENDOTHELIOTROPIC HERPESVIRUS (EEHV): AN UPDATE FROM THE USA

Lauren L Howard[1] and Sonja Luz[2]

[1] San Diego Zoo Safari Park, 15500 San Pasqual Valley Road, Escondido, CA 92027
[2] Wildlife Reserves Singapore, 729826 Singapore

Email: LHoward@sandiegozoo.org

Abstract

Elephant endotheliotropic herpesvirus (EEHV) causes severe, often fatal hemorrhagic disease in young Asian elephants (*Elephas maximus*). This virus has evolved with elephants over millions of years, and is carried and shed asymptomatically by most elephants tested over time.
Fatalities from EEHV Hemorrhagic Disease, or EEHV HD, have been documented in Asian and African elephants (*Loxodonta africana*) under human care, and in free ranging Asian elephants.

As of January 2017, there have been 33 cases of EEHV HD, with 24 fatalities and 9 survivors, in Asian elephants born in North America since 1980. In a 2015 survey of the N American elephant population, EEHV was the cause of death of 40% of all deaths in Asian elephants between 1 and 37 years old, making it the single greatest cause of death in Asian elephants born in North America. Hemorrhagic disease associated with EEHV occurs most commonly in Asian elephants between 1-8 years of age. An epidemiologic study in North America, presented in 2013, demonstrated no significant risk factors for EEHV HD, including genetics, exposure to African elephants, or management style.

EEHV HD can be diagnosed by PCR of whole blood or tissue collected from an affected elephant. The presence of the virus in the blood, or viremia, may be a subclinical occurrence or could indicate serious, even fatal, disease. Research has shown that Asian elephants may have subclinical, low level viremia for one to several weeks prior to the onset of clinical signs of illness.

In North America, it is recommended that young Asian elephants between 1 and 8 years old have blood collected weekly for EEHV PCR and to evaluate their CBC (complete blood cell count). The goal of this monitoring is to help calves survive potentially fatal infection through early detection and aggressive treatment.

Treatment recommendations for elephants with EEHV HD have been developed and shared internationally. Detailed treatment recommendations can be found on www.eehvinfo.org. Principles of treatment for EEHV HD remain the same across North America, Europe, and Asia, though subtle regional differences exist. Early detection of viremia is important to allow early initiation of antiviral and supportive treatment. Elephants that are suffering from EEHV HD may not appear very ill, even as they are developing severe internal hemorrhage. Therefore, aggressive, early treatment, sometimes initiated before a diagnosis can be confirmed, is the veterinarian's best chance in helping a calf survive this terrible disease.

The EEHV Advisory Group has Regional Steering Committees in North America, Europe, and Asia, and is available with further information on EEHV and advice on all aspects of management, testing, treatment, and monitoring. The EEHV Advisory Group website, www.eehvinfo.org has current information for veterinarians, researchers, and elephant care specialists, and is updated regularly by subject matter experts. Sample EEHV treatment protocols and a large bibliography are just two of many resources available on the website.

ELEPHANT ENDOTHELIOTROPIC HERPESVIRUS (EEHV) IN ASIA: UPDATES FROM THE ASIAN EEHV WORKING GROUP

Sonja Luz[1] and Lauren L Howard[2]

[1] Wildlife Reserves Singapore, 729826 Singapore
[2] San Diego Zoo Safari Park, 15500 San Pasqual Valley Road, Escondido, CA 92027

Contact: sonja.luz@wrs.com.sg

Abstract

Since it was first discovered in 1995, Elephant Endotheliotropic Herpesvirus Hemorrhagic Disease (EEHV-HD) has become the largest single cause of death in captive Asian elephants. While most of the confirmed cases have been reported from North American and European Zoos, fatal infections with EEHV are now being diagnosed with increasing frequency in both managed and wild elephant populations in Asia. To raise more awareness and inform regional stakeholders about currently available knowledge and best practice and to better coordinate research in a critical region, a first EEHV-Asia meeting was organized in Singapore in 2015. The goals of this first workshop were to (1) invite and link up international experts to build up the regional capacity and provide a comprehensive update on the EEHV; (2) To develop a region-specific strategy including short-term and long-term goals for research, disease management (i.e. prophylaxis, diagnosis and treatment) and funding; (3) To promote collaboration and coordination by creating a supportive EEHV network to efficiently manage EEHV in Asia. The 2 key outcomes from this meeting was the formation of the Asian EEHV Working Group and the printed guidelines for the management of EEHV in Asia.

At the second Asian EEHV Working Group Meeting in November 2016, a total of 68 cases of EEHV HD were identified among the delegates of 8 Asian elephant range countries, with 65 of the cases resulting in death and 12 of the deaths identified in free ranging elephants. The first published, PCR confirmed case of EEHV in Asia was reported in Cambodia in 2006 (Reid et al., 2006). More recently, publications have documented a fatal EEHV1 infection in Laos (Bouchard et al., 2014), fatal EEHV1 and EEHV4 infections in Thailand (Sripiboon et al., 2013), and fatal EEHV1 infections in both free ranging and captive elephants in India (Zachariah et al., 2013). Healthy Asian elephants under human care in India were shown to shed EEHV1, EEHV4, and EEHV5 from their trunks in 2014 (Stanton et al., 2014) a finding very similar to what is seen in Asian elephant herds in North America (Stanton et al., 2010) and Europe (Hardman et al., 2012). The 26 workshop participants (from 7 Asian countries,

the US and Europe) attending the 2nd Asian EEHV meeting voted on a list of 21 areas of research and outreach that priority in Asia should be given to the evaluation of EEHV Epidemiology and Risk Factors, the establishment of cPCR and qPCR EEHV Laboratories and the surveillance of wild elephants for EEHV.

With that the next step is to hold an EEHV diagnostics training workshop in Asia at Kasetsart University, Thailand in November (tentative dates: 15-17th November 2017), to train additional trainers in EEHV qPCR and cPCR diagnostic. The 3rd Asian EEHV Working Group meeting is currently scheduled for the 18th of November.

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ELEPHANT ENDOTHELIOTROPIC HERPESVIRUS (EEHV): AN UPDATE FROM THE EUROPE, FEBRUARY 2015 – MAY 2017

Willem Schaftenaar

Rotterdam Zoo, Rotterdam, The Netherlands

Contact: w.schaftenaar@rotterdamzoo.nl

Abstract

Since the previous workshop in Houston (February 2015), 7 calves have died of EEHV-HD in Europe, 6 cases of EEHV-1A and 1 case of EEHV-1B (Table below).

Date	Zoo	Name	EEHV-type	Age	Remarks
04-06-2015	Whipsnade	Max	EEHV1A	1 yr 7 mo	Fatal EEHV-HD[1]
15-09-2015	Chester	Bala Hi Way	EEHV1A	2 Yr 6 Mo	Fatal EEHV-HD
02-11-2015	Chester	Hari Hi Way	EEHV1A	3 Yr	Fatal EEHV-HD
28-11-2015	Le Pal	Jade	EEHV1B	7 Yr 6 Mo	Fatal EEHV-HD[2]
07-12-2015	Amsterdam	Mumba	EEHV1A	4 Yr 5 Mo	Fatal EEHV-HD
23-01-2016	Ostrava	Sumitra	EEHV1A	2 Yr	Fatal EEHV-HD
15-03-2016	Estepona	Buba	EEHV1A	2 Yr 10 Mo	Fatal EEHV-HD[3]
08-12-2015	Le Pal	Tom	EEHV1	1 Yr 9 Mo	Symptoms, survivor
15-03-2017	Kolmarden	Namsai	EEHV1B	3 Yr 9 Mo	Symptoms, survivor

[1]High antibody levels in May and Jun 2015
[2]No antibodies in November 2015
[3]99% nucleotide similarity with the Chester zoo case Jamilah (born at Chester Zoo)

Other activities:

- Recommendations on monitoring of EEHV in calves in Europe were drawn in mid-2015.
- The drafted recommendations were approved by the Elephant TAG in early 2016.
- A European EEHV workshop was held in Rotterdam in May 2016.

- On the final day of the Houston workshop, a group of European participants expressed the wish to form a European EEHV Research Steering Committee. This was briefly discussed again at the Rotterdam Workshop. In December 2016, Tim Bouts (EEHV research coordinator), Willem Schaftenaar and Imke Lüders (International EEHV-Advisory Group) and Fieke Molenaar (zoo vet at ZSL Whipsnade Zoo) met in Rotterdam to explore the possibilities to form such a steering group. The group aims were to define projects in cooperation with the current research groups and to explore funding opportunities from the European Union. The steering group is presently gathering advice on subsidy and funding applications.

- Currently a proposal has been made by Catalyze, a company that aims to accelerate the development of new products and applications in the field of life sciences and biotechnology.

EEHV IN NEPAL: HISTORY, TREATMENT AND SURVEILLANCE

Amir Sadaula[1], Prasad Gairhe K[2], Luitel H[3], Prasad Pokheral C[1]

[1]National Trust for Nature Conservation Biodiversity Conservation Center, Sauraha, Chitwan, Nepal
[2]Chitwan National Park, Chitwan, Nepal
[3]Agriculture and Forestry University, Rampur, Chitwan, Nepal

Contact: naturalamir@gmail.com

Abstract

Nepal has a population of 220 captive elephants, along with more than 100 wild elephants, distributed in three fragmented habitats. In captive herds, 3 – 4 calves are born each year. Nepal has experienced 8 clinical cases of EEHV since 2002 with age range between 1 to 3 years. All these cases occurred during June to December, with 7 cases since 2009. Among the 8 cases, 2 calves survived among 3 treated with antiviral drugs (2 survived - Famciclovir, 1 dead - acyclovir).The drug administration in a recent survival case in December, 2015 treatment started with clinical sign was as follows: Famciclovir – 12 mg/kg BW per rectal QID for 2 days, TID for next 2 days and BID from 3rd to 21st days, supportive drugs such as furosemide from 3rd to 5th day, vitamin B complex, antibiotics and including fluid therapy (new Nepal Protocol). Screening surveillance for EEHV1 excretion was carried out in 10 breeding cows in August 2016. Trunk washes and conjunctival swab samples were collected weekly for 4 weeks and tested using a conventional PCR. Seven out of 40 samples (6 out of 10 elephants) tested positive for viral DNA, indicating a high prevalence of the virus. The majority of cases in Nepal are seen in isolated herds with a single calf. It could be hypothesized the calves in isolated herds exposed to an abrupt high dose of virus following disappearance of maternal antibody whilst calves living in a multiple age group herd this exposure is gradual and of low virus load.

Acknowledgement

We would like to thank Kiran Rijal and Grihamani Nepal for their help in treating of the EEHV case.

UPDATE ON EEHV IN THAILAND: THE ROLE OF A NEW EEHV TASKFORCE

Khajohnpat Boonprasert[1], Angkawanish, T.[2], Sombutputorn, P.[2], Langkaphin, W.[2], Monchaivanakit, N.[2], Somgird, C.[1], Bansiddhi, P.[1], Sriphiboon, S.[3] and Titaram, C.[1]

[1] Center of Excellence in Elephant Research and Education, Chiang Mai University, Thailand
[2] Thai Elephant Conservation Center, National Elephant Institute, Forest Industry Organization, Thailand
[3] Faculty of Veterinary Medicine, Kasetsart University, Bangkok , Thailand

Contact: khajohnpat@gmail.com

Abstract

Since 2008, 32 cases of Elephant Endotheliotropic Herpesvirus (EEHV) have been molecularly confirmed in Thailand, mostly in juvenile elephants aged between 1-10 years old. This fatal hemorrhagic disease has caused the death of Thai elephant calves at a rate of 1-2 cases per year and remains the most serious health threat to the elephants in Thailand. The clinical signs included severe lethargy, facial edema, high fever, tongue cyanosis and sudden death, and most cases died within 24 h of showing the initial signs, either without treatment or following a few days of treatment. Three cases have recovered.

In Thailand, first official survival case report was published in "the 10th international EEHV meeting in Houston,Texas, USA, 2015". The 2-year-old male elephant showed classical signs of EEHV infection, and EEHV type 1A was confirmed using conventional PCR. Acyclovir was used to treat this case alongside antibiotics, fluid therapy and supplementary support, including 24-hour intensive veterinary care. On days 8 and 9 of treatment, the elephant began to show signs of recovery followed by decreased tongue cyanosis, decreased facial edema and increased activity. Clinical signs continued to improve with treatment and resolved by day 11.

A 'double-type' infection was detected in January 2016 in the southern part of Thailand. This was in a 3-year-old female elephant that had been sent to the Southern Elephant Hospital with initial signs of anorexia and depression for the previous 2 days. The elephant received intensive veterinary care and continuous treatment for 34 days before she died. Serial blood samples were collected daily from day 1 of admission until death. Samples were sent to the diagnostic laboratories in Chiang Mai University and Kasetsart University for molecular confirmation and viral load measurement by qPCR. The results indicated that the elephant was infected with EEHV types 1A and 4. Acyclovir and famciclovir were used with standard EEHV supportive treatment protocols. Gross and histopathological findings revealed severe internal organ damage with marked sub-acute chronic multifocal multiorgan necrosis. The presence of the *Clostridium perfringens* toxin gene indicated secondary bacterial infection.

In October 2015, an EEHV Taskforce was established by The Thai Elephant Conservation Center of The National Elephant Institute, Forest Industry Organization (TECC-NEI) in

collaboration with the Faculty of Veterinary Medicine, Chiang Mai University. This group serves to update stakeholders on the concurrent EEHV disease status in Thailand, and establishing a network to share knowledge, experiences, and case management. A meeting of the Taskforce held in February 2016 resulted in the publication of EEHV Monitoring Guidelines for Mahouts, Camp Managers, and Veterinarians. Future aims of the Taskforce include further training and education, as well as continuing applied research into the treatment and management of EEHV.

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DIFFERENT EVOLUTIONARY HISTORIES OF THE TWO CAPTURED HOST VOX2 GENES IN EEHV GENOMES

Zong J, Long SY, Heaggans SY and **Gary S Hayward**
Johns Hopkins School of Medicine, Baltimore, Maryland USA

Contact: ghaywar1@jhmi.edu

Abstract

The mammalian OX2(CD200) ligand is an immunoglobulin(Ig)-family membrane protein expressed in monocyte-macrophages that functions via cell-to-cell surface interactions with the OX2 receptor protein to play a role in dampening host inflammatory responses to infections. Two separate capture events of host vOX2 genes have occurred during evolution of the Deltaherpesvirus sub-family and both display remarkable novel features. One of these referred to as "new" E54(vOX2-1), is present in an unspliced form in EEHV1, EEHV2, EEHV5 and EEHV6 and displays 20% DNA divergence from the host version, indicating that it was captured more than 20 million years ago. Despite this, the viral proteins retain between 90 and 97% identity to the entire 298-aa Ig-v plus Ig-c domain encoded by exon3 plus exon4 of the host elephant OX2 protein, with nearly all the nucleotide changes representing highly selected synonymous mutations. On the other hand, the other "old" vOX2 gene (E23/E24/E25) evidently retained the host exon3-exon4 splice junctions and underwent gene duplication or triplication events in EEHV1 and EEHV5, while also acquiring a high level of divergence and multiple additional alternative splicing signals. Furthermore, in EEHV1 (but not EEHV5) one version (vOX2-3) has become a soluble secreted form with an acidic C-terminal tail replacing the usual hydrophobic transmembrane anchor domain.

To understand more about these processes and examine the level and nature of strain differences here, we have PCR amplified and sequenced both gene blocks (880-bp and 3.4-kb) directly from pathological DNA samples from 40 different Asian elephants from North America, Europe or India (29x EEHV1A, 5x EEHV1B and 6x EEHV5) that had acute hemorrhagic disease (HD) or were asymptomatic virus shedders. The results revealed that both loci display high levels of nucleotide hypervariability that is largely unlinked to the standard A and B subtyping patterns that occur in the CD-I, CD-II and CD-III chimeric domains in the central conserved region of these genomes. Many EEHV1A strains differ in vOX2-1 by up to 15% at the nucleotide level in patterns that are too complex to assign as subtypes, although there are also multiple examples of both epidemiologically related as well as non-epidemiologically related identity as well. For vOX2-2/3/4 in EEHV1, all four of the duplicated vIg-v and vIg-c domains display multiple clustered subtypes, but they are often scrambled and unlinked between the adjacent domains. Furthermore, three strains show significantly greater divergence plus altered exon structures from all the others, with two identical ones referred to as overall E23/24/25 block B-subtypes which does seemingly link with the previous chimeric B-subtyping, and a third that is sufficiently different from both the A and B subtypes to be designated as a novel C-subtype in E23/34/25. Finally, for

EEHV5, the five individual A-subtype examples show variable levels of divergence and are different from the single known B-subtype, but unexpectedly two of them proved to be fragmented (i.e. having multiple terminators and frame shifts that must inactivate one of the three adjacent highly spliced “old” vOX2 genes.

The captured vOX2 regions in EEHV display some of the highest levels of strain divergence found in any herpesvirus genomes. Both the extremely high level of amino acid conservation in vOX2-1 (not seen in any other captured herpesvirus genes, including other vOX2s) and the presence of otherwise rare complicated splicing patterns in the “old” vOX2-2/3/4 block strong imply that they likely play important but as yet unknown roles in the biology of at least the AT-rich branch of the *Proboscivirus* genus. The latter “old” duplicated vOX2 proteins especially also appear to be subject to enormous “active” evolutionary flexibility and we suspect that they might represent either immediate-early or latency genes.

Session 3: Research updates

GENETIC DIVERSITY AMONG EEHVS FROM FATAL AND LATENT INFECTIONS OF ASIAN ELEPHANTS IN EUROPE

Hannah Davies[1], Seilern-Moy K[2], Steinbach F[1,2], Dastjerdi A[2]

[1]University of Surrey, Guildford, GU2 7XH, United Kingdom
[2]Animal and Plant Health Agency (APHA), Weybridge, KT15 3NB, United Kingdom

Contact: akbar.dastjerdi@apha.gsi.gov.uk

Abstract

Elephant Endotheliotropic Herpesviruses (EEHVs) are the leading cause of a haemorrhagic disease in the endangered Asian elephants (*Elephas maximus*). The viruses have caused multiple fatalities in juvenile Asian elephants in Europe and have also been detected in trunk, conjunctiva and oral mucosa secretions of healthy but latently infected elephants. These viruses have primarily been genetically typed using limited sequence data. However, their genetic diversity and relatedness to other viruses detected from similar cases at the same holdings, across Europe or globally are vastly unknown.

In this study we have investigated a selection of EEHV gene sequences both relatively conserved and substantially different across between isolates. For this, DNA polymerase (POL), Glycoprotein L (gL), gH, gB and Thymidine Kinase (TK) complete gene sequences of EEHVs detected in eleven fatal and latent cases were analysed, either by Sanger or Next Generation Sequencing. The samples analysed here had been identified positive for either EEHV-1A or -1B. Within one holding, two of the viruses detected were identical in their DNA sequences. Intriguingly, the two fatal cases with identical EEHV-1A DNA sequences occurred years apart, while the zoo experienced further cases with different gene signatures in between. All further EEHVs analysed were genetically different both within those analysed here and towards those described in GenBank.

Acknowledgement
We thank numerous European zoos for the provision of materials. Financial support provided by the Zoological Society of London (ZSL), Chester Zoo and Woburn Safari Park is also highly appreciated.

EXPLORING THE INTERFERON SYSTEM OF ASIAN ELEPHANTS (*ELEPHAS MAXIMUS*) FOR CONTROLLING ELEPHANT ENDOTHELIOTROPIC HERPESVIRUS INFECTIONS

Jonathan Haycock[1,2], Steinbach F[1,2] and Dastjerdi A[1]

[1] Animal and Plant Health Agency (APHA), Weybridge, KT15 3NB, United Kingdom
[2] School of Veterinary Medicine, University of Surrey, Guildford, GU2 7XH, United Kingdom

Contact: akbar.dastjerdi@apha.gsi.gov.uk

Abstract

The apparent lack of efficacy for the currently applied antiviral treatments and the inability to culture EEHVs *in vitro* have hindered clinicians and virologists in their search for efficient prophylactic and therapeutic approaches to clinical EEHV infections. It is known that both the innate and adaptive immune responses play a critical role in controlling herpesvirus infections and the interferon (IFN) system is increasingly being investigated in humans as an alternative to conventional therapies for a wide variety of viral diseases.

This study has begun to investigate the Asian elephant (*Elephas maximus*) innate immune system testing the hypothesis that EEHV infections are mediated at least in part by IFN signalling pathways. Using the Asian elephant genome sequence reads, predicted gene sequences for IFN alpha (α), beta (β), gamma (γ) and lambda (λ) were assembled, including multiple non-allelic members and a single pseudogene from the IFNα gene family. IFNα and IFNβ genes were then cloned into a bacterial expression vector and the recombinant proteins were successfully expressed. Optimisation of IFN purification is currently on going. Upon purification, the anti-viral activity of the recombinant IFN will be assessed *in vitro*, using other viruses. If proven successful, the project will then address issues related to quality control and standardisation of the product. In addition, the recombinant IFNs could be used for the development of assays to investigate the dynamics of IFNs in healthy and diseased elephants e.g. those from EEHV disease cases. Administration of therapeutic agents that produce IFNs in a clinical scenario remains a potential eventual outcome of this work.

Acknowledgments

Funding for this project is partly provided by Zoological Society of London (ZSL), Chester Zoo and Woburn Safari Park. Many thanks to the elephant team at ZSL Whipsnade Zoo for the provision of samples.

THE HOST RESPONSE TO ELEPHANT ENDOTHELIOTROPIC HERPESVIRUS INFECTION

Kathryn L Perrin[1,2], Kristensen AT[1] and Bertelsen MF[1]

[1]Center for Zoo and Wild Animal Health, Copenhagen Zoo, Roskildevej 38, 2000 Frederiksberg, Denmark
[2]Institute for Clinical Veterinary Medicine, University of Copenhagen, Dyrlægevej 16, 1870 Frederiksberg C, Denmark

Contact: kap@zoo.dk

Abstract

Copenhagen Zoo and the University of Copenhagen are supporting a three year PhD project examining the host response to Elephant Endotheliotropic Herpesvirus (EEHV) infection. We hypothesise that there will be significant differences in the host response to EEHV viraemia that determine the outcome – survivor or fatality.

Immunogenetics: In order to test the hypothesis that there will be significant differences in immunogene variability between elephants which survive to adulthood and those that succumb to EEHV, DNA samples from approximately 100 individuals were obtained from institutions in Europe and North America. Currently these samples are undergoing PCR analysis prior to sequencing to examine variability of major histocompatibility complexes and Toll-like receptors. This work is in collaboration with Alex Greenwood at the Leibniz Institute for Zoo and Wildlife Research in Berlin, Germany.

Pathology review: A comprehensive pathology review is being performed on fatal cases in Europe and North America. Existing records and tissues have been requested via the EEHV Advisory Group pathology advisor, Daniela Denk, and the review is performed in collaboration with Dr Denk at the International Zoo Veterinary Group, UK.

Immunology and clinical pathology: Characterization of coagulation parameters of healthy Asian elephants has been undertaken and is currently being published,[1] and investigations into coagulation parameters in clinical cases of EEHV have been initiated.[2] Citrated plasma and serum samples have been collected from several clinical cases to date, and prospective collection has been requested from viraemic calves. We hypothesise that there will be differences in the host response (haemostatic, inflammatory and immunological) between calves that survive EEHV viraemia and those that succumb.

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GENETIC ANALYSIS OF COAGULATION FACTOR VII IN FATALITIES CAUSED BY EEHV-HD IN ASIAN ELEPHANTS *(Elephas maximus)*

Sonia J Fontes, Fickel J and Hildebrandt TB

Leibniz Institute for Zoo and Wildlife Research (IZW), Alfred-Kowalke-Str. 17, 10315 Berlin, Germany

Contact: fontes@izw-berlin.de

Abstract

Elephant endotheliotropic herpes virus (EEHV) is responsible for several fatal cases in the Asian elephant captive population, mainly in calves. One of the most common pathological findings in all necropsy cases is an acute haemorrhagic syndrome with the presence of pericardial effusion as well as micro haemorrhages in histological sections of several organs (Richman et al., 2000). Preliminary data presented by Bennett et al. (2015) showed that the majority of these EEHV deaths are clustered in groups of related captive animals. Lynch et al. (2017) reported that one captive adult male living in Australia, asymptomatic for bleeding tendency was found to have coagulation Factor VII (FVII) deficiency, with a single homozygous point mutation. Three of his offspring were also found to be heterozygotes for this mutation.

Our study aims therefore at a better understanding if an involvement of a hereditary coagulation disorder can be present in the onset of this haemorrhagic disease in the European population of Asian elephants.

Frozen tissue (liver and myocardium) or blood (EDTA tubes) samples were used to analyse coagulation FVII gene for mutation in four EEHV – HD fatal cases (1.3). From a total of 8 exons of the FVII gene, we successfully amplified and sequenced 6 exons, to investigate the presence of point mutations, removing the remaining 2 exons from this study.

We hypothesize that if Factor VII is correlated with EEHV-HD, then most likely all dead cases reported in Europe should present the same mutation pattern. We found two exons with variation within the investigated individuals. Exon 1, presented one single nucleotide polymorphism (SNP), where three out of four individuals presented identical homozygous sequences (G), and one individual showed a heterozygous (C/G) position. Another SNP was found in exon 6, where three out of the four individuals presented C/G polymorphism and one individual only a G (homozygote). For the other exons, no heterozygosity was detected and all sequences were identical among all individuals.

Although establishing a causative relation between Factor VII mutation and EEHV-HD is not yet possible, the next important step will be to test survivors of EEHV infected cases (that were submitted or not to antiviral treatment) for comparison. Our results might have important implications for future research, because if adults have the susceptible genotype and show high titers of the virus, but no EEHV disease related symptoms, it can suggest that

they were in contact with some element that protected them during growth and allowed them to achieve adulthood, presenting at this stage better chances of survival.

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THE EUROPEAN EEHV RESEARCH STEERING GROUP

Willem Schaftenaar[1], Fieke Molenaar[2], Tim Bouts[3] and **Imke Lüders**[4]

[1]Rotterdam Zoo, Rotterdam, The Netherlands
[2]Zoological Society of London, ZSL Whipsnade Zoo, Dunstable, Bedfordshire LU6 2LF, UK
[3]Pairi Daiza Zoo, Domaine de Cambron, B-7940 Brugelette, Belgium
[4]Geolifes, Frohmestr.7, 22457 Hamburg, Germany

Due to various countries and languages, communication is not as easily possible within Europe compared to the United States. There, research and diagnostics appear much better coordinated. In order to become better organized amongst European zoos and research institutions in respect to EEHV case records, EEHV research project coordination, veterinary care and the dissemination of latest updates, it was decided during the first European EEHV research workshop in Rotterdam in May 2016 that a European steering committee dedicated to EEHV related topics would be set up.

The idea is to have only members within this committee with a clinical/zoo background and no own research activities, in order to avoid conflict of interest. The vision is to prevent further elephant calves to succumb to EEHV by reinforcement of certain measures and projects. Our goals are:

- define research areas and set up a research strategy for Europe
- coordination of research fields (prevent overlap and encourage complementation of research between institutions – openness)
- assess scientific value and validity of research proposals
- help to raise funds for research projects
- help to set up qpCR in each EU country with Asian elephant breeding
- coordinate a tissue bank/database (sample portal) of both infected and unaffected elephants that can be distributed to researchers (with legal requirements for shipping samples)
- disseminate information treatment updates to clinicians

During an initial meeting in December 2016 at Rotterdam Zoo, the four authors agreed to take these tasks on. Initial steps are to receive approval by the elephant TAG and possibly the EAZA, translate and disseminate the EEHV management guidelines brochure prepared by Sonja Luz after the 1st Asian EEHV Strategy Meeting into several languages and modify these for EU zoo needs.

We hope that through the European EEHV Steering Group future research can be better planned and carried out, that financial support can be given and access to samples may be improved. The committee should act on behalf of and connect the scientific and the zoo veterinary community, and improve the overall communication amongst involved parties.

Session 4: Subclinical and latent EEHV

GENE TREES AND VARIANTS; MAKING BIOLOGICAL SENSE OF VIRAL GENOMICS

Judith Breuer

University College London (UCL)

IDENTIFYING T CELL RESPONSES TO ELEPHANT ENDOTHELIOTROPIC HERPESVIRUS (EEHV) PROTEINS IN ASIAN ELEPHANTS LATENTLY INFECTED WITH EEHV

Angela Fuery[1], Rongsheng Peng[1], and **Paul D Ling**[1]

[1]Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas, USA

Contact: pling@bcm.edu

Abstract

Elephant Endotheliotropic Herpesvirus (EEHV) can cause a devastating hemorrhagic disease in juvenile Asian elephants, often leading to death. Since adult elephants can be latently infected with the virus without succumbing to lethal infection, it is likely that the vulnerability of juvenile elephants is related to their poorly primed immune system, particularly the cellular compartment. Until now, there have been no studies of the Asian elephant T cell response, so the development of assays to investigate the T cell response of Asian elephants in this study has allowed us to screen EEHV proteins as potential vaccine candidates. We first developed an IFN-γ ELISpot assay using Rabies, a known antigen which was measured following routine vaccination of the elephant herd. Using this assay, we screened 7 elephants in a captive herd with evidence of prior infection with EEHV1A and observed robust responses to the EEHV1A Major Capsid Protein and glycoprotein B, where glycoprotein B induced significant IFN-γ responses in 5 of 7 elephants studied. Following production of an Asian elephant CD8-specific monoclonal antibody, we were able to analyse these responses further to find that IFN-γ responses to EEHV are associated with both CD8 and CD4 T cell populations. These studies have identified EEHV-specific T cells in Asian elephants for the first time, and have made the first steps to understanding the T cell priming required to protect against devastating EEHV disease.

SUBCLINICAL EEHV INFECTION: HAEMATOLOGICAL CHANGES AND ASSOCIATED ANTIBODY RESPONSE

Fieke Molenaar[1], Akbar Dastjerdi[2] and Petra van den Doel[3]

[1]Zoological Society of London, ZSL Whipsnade Zoo, Dunstable, Bedfordshire LU6 2LF, UK
[2]Animal and Plant Health Agency (APHA) Weybridge, KT15 3NB, United Kingdom
[3]Erasmus Medical Centre, 3015, Rotterdam, The Netherlands

Contact: fieke.molenaar@zsl.org

Abstract

A 2.5 year old male Asian elephant tested positive for EEHV1 by a qPCR, viral load of 2,500 vge/ml of blood, on a routine EEHV testing in February 2017. There was no evidence of clinical signs and the calf behaved completely normal. Daily EDTA blood and serum samples were subsequently taken to monitor EEHV load, haematology, and quantitative serology. Daily citrate bloods were also taken from Day 6 of viraemia to analyse for coagulation factors.

From Day 1 to 14, the viraemia fluctuated between detection level and 2,500 vge/ml. On Day 1 of viraemia, in-house evaluation of a blood smear showed a subjectively low WBC count, decreased monocytes and platelets, a relative increased heterophils and low normal lymphocytes, compared to his individual reference range. The WBC recovered to low normal on Day 3 and elevated from Day 4 for two weeks before returning to normal range. Platelet numbers return to normal on Day 4. After the initial marked dip, the monocytes count returned to low normal on Day 3 and were slightly elevated on Day 4, but with marked elevation on Day 5 and 6. There was a relative dip in monocytes on Day 7 and 12. Lymphocytes were seen in normal range on Day 1-5, but with a significant decrease on Day 6. Markedly elevated counts were observed on Day 7-10, with a dip on Day 11, and elevation again on Day 12-13. Lymphocyte counts returned to normal on Day 14, but showed a sudden relative increase on Day 25. They returned back to normal thereafter.

Throughout 2016, this particular calf produced consistently high EEHV antibody levels that appeared to increase just after major life events, such as the arrival of a new calf, delivery of transport crates to the barn and departure of two of the herd's male juveniles. Just prior to the EEHV viraemia in February, antibody levels were found to be lower than the previous year levels. Once the viraemia commenced, the antibodies dip even further on Day 6 and 7, but the levels recuperate on Day 8 and remained at this level until Day 14. Levels of antibodies detected appear to be trailing the rise and fall of lymphocytes seen on haematology.

Haematological response to subclinical EEHV1 infection in this particular case highlights the role of monocytes in controlling the infection during the first few days of viraemia, followed by lymphocyte and antibody response.

ASSESSMENT OF A LANCET-AND-SWAB BLOOD SAMPLING TECHNIQUE FOR SURVEILLANCE OF ELEPHANT ENDOTHELIOTROPIC HERPESVIRUS INFECTION

Jonathan Haycock[1,2], Lopez J[3], Mckenzie A[3] and Dastjerdi A[1]

[1] Animal and Plant Health Agency (APHA) Weybridge, KT15 3NB, United Kingdom
[2] School of Veterinary Medicine, University of Surrey, Guildford, GU2 7XH, United Kingdom
[3] North of England Zoological Society, Chester, CH2 2LH, United Kingdom

Contact: j.lopez@chester.org

Abstract

Detecting and monitoring viraemia is a key element in the management of Elephant Endotheliotropic Herpesvirus (EEHV) infection. However, both the management system employed and an individual animal's compliance can impose challenges for standard venepuncture. A modified lancet bleeding method (Lopez at al., 2015) and several blood sampling matrices were evaluated in this study for routine EEHV surveillance in Asian elephants (*Elephas maximus*). The procedure enabled weekly sampling from elephants as young as nine months of age. The blood sampling matrices were evaluated for their relative sensitivities in measuring β-actin, TNFα and/or EEHV-1 by qPCR. Foam swabs (Sigma Aldrich Catalogue #: A9601-100EA) and flocked swabs (VWR Catalogue #: SLIN502CS01) both produced significantly ($P < 0.05$) lower quantitation cycles (Cq) and were thus deemed more sensitive than filter papers, FTA cards, or conventional cotton-tipped swabs. The two swab types also demonstrated comparable sensitivity for the detection of EEHV-1 to that of a similar volume of EDTA whole blood. EEHV-1 viraemia was repeatedly detected in two Asian elephant calves by means of lancet-and-swab samples and in one animal, five days prior to the development of clinical signs. The sensitivity of this method and early detection of viraemia are most likely to be compromised by a low blood yield from the lancet application. Therefore, standard venepuncture remains the recommended blood sampling method and training for such should continue to be the priority. However, this lancet-and-swab technique offers a suitable alternative for EEHV surveillance in situations where venepuncture may not be practical.

Acknowledgments
Chester Zoo for veterinary support and to elephant keepers at both Chester zoo and ZSL Whipsnade zoo for sample collection. Prof. Falko Steinbach, APHA, for his constructive comments on the study and Dr. Jeewaka Mendis, University of Surrey, for support with the statistical analysis. Funding for this work was partly provided by the Zoological Society of London (ZSL), Chester Zoo and Woburn Safari Park.

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Session 5: Practical treatment and animal access

ASIAN ELEPHANT (*ELAPHAS MAXIMUS*) JUVENILE SURVIVING EEHV HEMORRHAGIC DISEASE IN KOLMARDEN ZOO, SWEDEN

Louise Guevara[1] , Moller T[1], Perrin KL[2], Dastjerdi A[3]

[1]Kolmarden Zoo – Kolmarden 618 92, Sweden
[2]Copenhagen Zoo, Roskildevej 38, 2000-Frederiksberg, Denmark
[2]Animal and Plant Health Agency (APHA) Weybridge, KT15 3NB, United Kingdom

Contact: louise.guevara@kolmarden.com

Abstract

In March in 2017, the nearly four year old male Asian elephant (*Elephas maximus*) at Kolmarden Zoo Sweden was observed to be slightly lethargic, associated with social stress within the herd. Four days later two minor lesions developed in the palate, prompting blood collection for PCR analysis at the APHA. The following day the calf showed slight cyanosis at the edges of the tongue. Treatment was started immediately with acyclovir (15mg/kg BID, Aciklovir STADA, STADA Nordic ApS, Herlev, DK). Daily treatments were administered under sedation and included plasma and/or whole blood for four days, large volumes of rectal and intravenous fluids and butorphanol 0.008 mg/kg. Dexamethasone 0.05 mg/kg i.v. was given during the first two treatments. Antibiotic cover was provided throughout and blood culture identified *Clostridium glycolyticum.* Omeprazole 1 mg/kg and probiotics were given for three weeks.

The calf displayed early clinical signs of hemorrhagic disease which progressed to edema of the head and tongue, progressive cyanosis and obvious pain. Visible symptomatic improvements could be noted on the fifth day of treatment. Early blood samples showed a marked thrombocytopenia and leukocytosis with pronounced toxic heterophils. The monocyte level was stable at around 60% during the disease period. Within one week after onset of antiviral treatment the viral load started to clearly drop off and the total white blood cell count declined two days later. The thrombocyte count started to rise early after the therapeutic onset, proving the effect of this combined therapy.

CLINICAL INFECTION OF A YOUNG CAPTIVE ASIAN ELEPHANT WITH ELEPHANT ENDOTHELIOTROPIC HERPESVIRUS 1A IN THE BERLIN ZOO

Walid Azab[1], Andreas Ochs[2] and Klaus Osterrieder[1]

[1] Institut für Virologie, Robert von Ostertag-Haus, Zentrum für Infektionsmedizin, Freie Universität Berlin, Robert-von-Ostertag-Str. 7-13, 14163 Berlin, Germany
[2] Zoologischer Garten Berlin AG, Hardenbergplatz 8, 10787 Berlin, Germany

Contact: Walid.Azab@fu-berlin.de

Abstract

Elephant endotheliotropic herpesviruses (EEHVs) are a continuous threat for young Asian elephants. EEHV generally causes acute hemorrhages in you elephants, but some elephant calves have survived the infection. Here we present a confirmed laboratory infection of a young 5-year-old female Asian elephant (Anchali) in the Berlin zoo that underwent subclinical EEHV 1A infection. Initially, high EEHV 1A viral loads were detected in trunk swabs obtained from Anchali during routine screening of all elephants in the zoo. The young elephant showed no clinical signs except of slight irritability; however, the general condition remained unaffected. EEHV 1A was continuously shed for almost one year with fluctuations in viral loads from time to time. Two other adult elephants out of six showed viral shedding for short duration without obvious clinical manifestation. Anchali was treated with ganciclovir (GCV) 5 mg/kg. However, GCV showed no effect on viral loads or shedding. Our investigation further highlight the continuous threat of EEHV 1A in young captive Asian elephants and stress on the importance of routine monitoring of captive elephants to facilitate early detection of EEHV infection. It seems likely that ganciclovir did not cure the young elephant and it is unclear if treatment helped to avoid a fatal outcome. Finally, efforts need to be continued to detect suitable antiviral drugs and/or suitable treatments for EEHV 1A infection.

ELEPHANT ENDOTHELIOTROPIC HERPESVIRUS-5 (EEHV-5) VIREMIA AND MEDICAL MANAGEMENT IN AN ASIAN ELEPHANT CALF (*ELEPHAS MAXIUMUS*) AT THE HOUSTON ZOO

Christine Molter[1] and Martina Stevens[1]

[1]Houston Zoo, Inc., 1513 Cambridge Street, Houston, Texas, USA

Contact: cmolter@houstonzoo.org

Abstract

With a herd of breeding Asian elephants (*Elephas maximus*), the threat of elephant endotheliotropic herpesvirus (EEHV) is omnipresent at the Houston Zoo. Through a collaborative effort involving the elephant care and veterinary teams, in conjunction with the Baylor College of Medicine, a strategy has been developed for persistent monitoring, rapid detection, and aggressive treatments of EEHV viremia. Key elements include training calves for blood collection for weekly complete blood cell counts (CBC) and quantitative polymerase chain reaction (qPCR). Daily vital parameter and clinical signs are also monitored. When viremia is detected and treatment is indicated, immediate supportive care consisting of fluid and antiviral therapy is started. Treatments are continued until clinical signs resolve and viremia regresses.

In 2016, a 2 year old male Asian elephant developed EEHV-5 viremia with concurrent monocytopenia, temporal swelling, and hyperemic mucous membranes. The first notable sign of viremia was a rise in EEHV-5 viral genome equivalents (vge), as found on weekly qPCR screening, prior to CBC changes and clinical signs. The animal was sedated on 4 separate occasions during the viremic episode with detomidine and butorphanol for treatments, which included intravenous and rectal fluids, plasma transfusions, famciclovir, and antibiotics. Between sedations, the animal continued to receive rectal fluids and famciclovir. The animal's viral load increased and monocyte count decreased initially during treatment and did not rebound for 9 days. The animal recovered from this viremic episode and currently is clinically stable with no evidence of EEHV viremia for approximately 1 year.

Acknowledgements
The Houston Zoo elephant care team, Houston Zoo veterinary team, and Baylor College of Medicine are acknowledged for their contributions to elephant care and EEHV research.

EARLY EEHV VIREMIA DETECTION AND SUCCESSFUL TREATMENT OF SEVERE EEHV-HD IN A TWO YEAR OLD CALF

Charlie Gray[1] and Peter Rich[1] (Presented by **Imke Lüders**[2])

[1]Afrian Lion Safari, Ontario, Canada
[2]Geolifes, Frohmestr.7, 22457 Hamburg, Germany

Email: cgray@lionsafari.com, msah@rogers.com

In this talk, the course of events during an EEHV-HD case in a large breeding group of Asian elephants is described. Aggressive and immediate treatment resulted in survival of the calf in this case.

Two year old female Asian elephant calf "Anna Mae" (born 04/05/2015) is part of a group of 16 elephants at the African Lion Safari, Canada (ALS). In the group, currently, five other female calves are present at the age of 2-5 years. Since two fatal EEHV cases were confirmed prior to 2006, weekly blood sampling is now started by the age of 1 year for regular PCR monitoring.

On day 1, blood samples returned positive from PCR for Anna Mae. The calf was showing no clinical signs, the blood work appeared normal, the body temperature was at 37°C, body weight at 650kg. Oral famciclovir tablets were started that evening nevertheless. On day 3, she appeared stiff in her right front foot in the morning. Leucocytes had dropped from around 20 $x10^9$/L to only 7.1 x 10^9/L. Decision was made to start with plasma administration and the calf was sedated with detomidine and 300ml of plasma were given via ear vein at noon. Since oral administration of famciclovir became difficult, it was continued rectally.
The leg movement was better during the next day and she appeared normal otherwise. On day 6, she was still positive during PCR testing, furthermore a drop in platelet count was now noticed from 475 to 143 $x10^9$/L. Her third eyelids showed hyperemia. Thus, Anna Mae was sedated again [10 mg Detomidine (0.015mg/kg), 5mg Butorphanol (0.0077mg/kg)] in the evening for a second plasma infusion. This time 593 g were administered. On day 7 she seemed relatively fine, an enema for fluid administration was given at night. On day 8, 571g of plasma were administered again during sedation and two enemas were given during the day.

On day 9, the blood picture worsened, platelets dropped further, creatinine increased, sublingual hemorrhages were noticed for the first time and Anna Mae appeared duller than the days before. Intensive IV fluid and plasma therapy was started in the afternoon, a total of 276g of plasma, 750 ml of Hetastarch fluid solution (Voluven®) and 400 ml NaCL were given IV during sedation. Enemas were administered 6 times during the day and throughout the night. Furthermore, she was started on 2mg/kg Ceftiofur IM and 250mg Flunixine-Meglumine orally via syringe and tube.

On day 10, the head started to swell up. Anna Mae received again IV plasma (533g) and 100 ml of Voluven®, the IV line was more difficult to establish however and finally could not kept going. Enemas were given 7 times during the day and at night.
On day 11, the facial edema had worsened, to the point that the calf was unable to swallow or open her eyes. She was unable to control or use her trunk, when swelling was at most severe stage. The tongue had turned purple. The hematocrit was at 33 % at this stage. Another round of IV fluids were started at 5.00 p.m. During 1.5 hours, 614g of plasma and 1 L of NaCL solution were given. At the same time, furosemide (250mg IM) was injected to drain the facial edema. Another 6 enemas were given throughout the night.
On day 12, Anna Mae appeared a little better, the head edema decreased a little bit. She started to gain control over her trunk again and ate small amounts of hay. Enemas were continued 6 times over the day.

During days 13-16, the little female improved continuously. Platelets had increased to over 500x 10^9/L again. The HCT was down to 24%, possibly due a combination of previous red blood cell leakage and over hydration. The facial edema disappeared within three days, but a small ventral abdominal edema started to form on day 16. Anna Mae ate and drank well from now and thus, enemas were discontinued after day 17. The ventral edema regressed. Antibiotics were also given rectally (1375mg enrofloxacine) from day 16 and both antibiotics were continued until day 20. From day 14 to 24, a leucocytosis was present (up to 39 x10^9/L leucocytes), possibly as a sign of good immune response now. On day 27, blood PCR was negative for the first time for EEHV and rectal Famcilovir was stopped on day 28.
Blood parameter continued to improve with leucocytes now at the range prior to illness (17-20 x10^9/L), while red blood cells are still under recovery (around 3.5 x 10^{12}/L prior to illness, currently at 2.8 10^{12}/L). Otherwise the calf seems to be back to normal.
No other calf was positive for EEHV during the course of Anna Mae´s disease.
The immediate and aggressive treatment seems important when fighting EEHV-HD. A combination of several factors has resulted in a positive outcome in this case:

- Early detection of viremia prior to any clinical signs due to training for weekly blood samples
- Intensive care possible, which included regular blood sampling, medication (orally, rectally, intramuscular, intraveneously) and enemas
- Minimal handling stress, since used to be tied up, and only slight sedation for IV drips needed (the elephant was sedated only slightly and then asked to lay down on command on the side needed for IV placement of catheter in the bottom ear. Due to the position of the ear and low dose of alpha-2 agonists, IV line was possible to establish in all attempts.)
- Intensive hydration (IV, rectally) and multiple times of plasma administration

This case gives hope in the fight against EEHV-HD and highlights the importance of intensive management of elephant calves at critical age.

FUTURE PLANNING: CALF ACCESS IN PC SITUATIONS

Kathryn Perrin[1,2]

[1]Center for Zoo and Wild Animal Health, Copenhagen Zoo, Roskildevej 38, 2000 Frederiksberg, Denmark
[2]Institute for Clinical Veterinary Medicine, University of Copenhagen, Dyrlægevej 16, 1870 Frederiksberg C, Denmark

Contact: kap@zoo.dk

Abstract

Copenhagen Zoo is typical of many zoos who have moved from a free contact management system, to that of protected contact, which results in challenges with respect to calf training. Early calf compliance is probably the most important limiting factor for the implementation of weekly blood sampling regimes for EEHV PCR and haematological monitoring. After a short introduction, attendees will be invited to contribute to an open discussion on the challenges and successes of training calves in protected contact, particularly in the first year of life. What has been effective? What can be learned from institutions still managing their elephants in free contact? When is it realistic to expect consistent training compliance for blood sampling? Any ticks and tricks? Establishment of these training behaviours is essential for individual calf monitoring and treatment, and to enable further research into the pathogenesis of this devastating disease.

Session 6: Future options?

HUMAN HERPESVIRUS VACCINES WITH AN EMPHASIS ON CMV

Paul Griffiths

University College London, Gower Street, London WC1E 6BT

Contact: p.griffiths@ucl.ac.uk

Abstract

The aim of this talk is to outline progress with preparing vaccines against human herpesviruses and seek parallels with the veterinary situation with EEHV.

Of the eight herpesviruses that infect humans, we have a licensed vaccine for only one; human herpesvirus three; HHV3; Varicella-Zoster virus (VZV) which is an alphaherpesvirus. A live attenuated vaccine prevents primary infection (chickenpox). A second vaccine uses a higher dose of the same strain of VZV to overcome natural immunity and boost cell mediated immunity. It is given to the elderly to reduce the incidence and severity of secondary infection with VZV (zoster). This represents the first proven application in humans of immunotherapy (giving vaccine to an individual already infected with that same virus). A recombinant vaccine based on glycoprotein E of VZV has recently been developed as an alternative to the live attenuated zoster vaccine.

Cytomegalovirus is a betaherpesvirus. I will outline its multiple disease associations and discuss pathogenesis, including quantitative means of diagnosis. I will then summarise the clinical trials that have been performed to date, emphasising the potential for discovering effective vaccines by reducing the quantity of virus in body fluids (pharmacodynamics). In summary, there is encouraging information that both humoral and cell mediated immunity can be induced, that each response can help control CMV viral load and that each response can be boosted by additional doses of vaccine.

EPIDEMIOLOGY, DISEASE MANIFESTATION AND TREATMENT OF VIRAL HAEMORRHAGIC FEVER

Colin Brown

Hospital for Tropical Diseases, University College London Hospital, London, United Kingdom & King's Sierra Leone Partnership, King's Centre for Global Health, King's College London, and King's Health Partners, London, United Kingdom

Contact: colinbrown1@nhs.net

Abstract

Colin Brown is an honorary consultant in infectious diseases and medical microbiology at the Royal Free Hospital, with experience of managing Ebola Virus Disease (EVD) and Crimea Congo Haemorrhagic Fever (CCHF) patients in the high level isolation unit. He is mainly based at Public Health England with a portfolio on respiratory, vaccine preventable and emerging infections. He is also the Infectious Diseases advisor to King's Sierra Leone Partnership, who pioneered a model of assessing and diagnosing EVD patient in holding units in government hospitals in Sierra Leone, where he spent approximately four months during the 2013-2016 West African outbreaks. Colin will talk about the epidemiology, disease manifestation and treatment of haemorrhagic fever, and our increased understanding of various aspects of clinical syndrome, pathophysiology, and disease sequelae. He will talk about the role of conventional and novel therapeutic agents that have been used in West Africa and in Europe and the United States for EVD, as well as discussing the many remaining unknowns in disease management, despite nearly 30,000 cases. Where possible, he will discuss these advances in the context of elephant herpesviruses.

Session 7: Cross matching and practical haematology

CROSS MATCHING FOR ELEPHANTS

Christine Molter,[1] Maryanne Tocidlowski,[1] and Fieke Molenaar[2]

[1]Houston Zoo, Inc., 1513 Cambridge Street, Houston, Texas, 77030, USA
[2]Zoological Society of London, ZSL Whipsnade Zoo, Dunstable, Bedfordshire LU6 2LF, UK

Contact: cmolter@houstonzoo.org

Of all the recent clinical EEHV cases that have survived advanced haemorrhagic disease or viremia, such as the calves in Denmark and Canada presented earlier on at this workshop, intravenous administration of plasma in as early a stage as possible appears to have contributed to sustaining the calves through the worst stages of the disease.

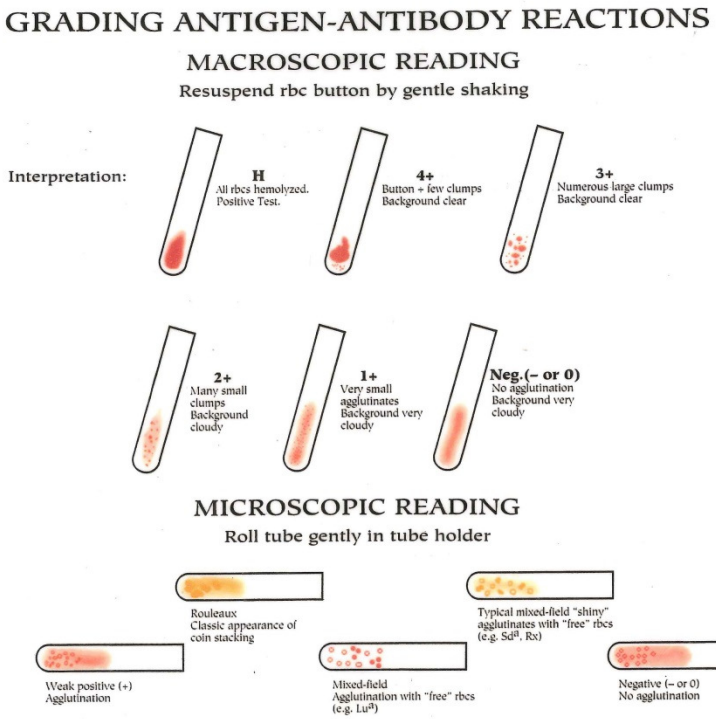
To be able to administer plasma in such cases, access to compatible donors, a -80°C freezer to bank sufficient plasma in advance or a refrigerator for holding fresh plasma, and the ability to place an intravenous cannula in the calf are essential.

To be able to give plasma safely, the blood of the donor should be compatible to that of the recipient as determined by using a minor cross match. To be able to give a whole blood transfusion safely, both a major and a minor cross match should be carried out. During this workshop we will describe a very basic way of cross matching that can take place in any veterinary clinic that has access to the necessary equipment. Cross matching can be time consuming (1-2 hrs). It is therefore prudent to carry it out at the time of plasma collection for banking, so that banked plasma is labelled with recipient calf suitability. This will save precious time during treatment of a clinical EEHV case. This cross matching technique can be used for any animal that needs a transfusion.

Materials required
1. EDTA (preferred) or serum tube (without the separator gel) from donor and recipient animals (all animals involved) 2. Centrifuge 3. Small clear tubes (glass preferred) for separating the plasma and for testing (estimate minimum 3 tubes/animal) 4. Physiologic saline (0.9% saline without preservatives). 5. Droppers or pipettes 6. Incubator 35-37oC 7. Markers for labelling tubes 8. Paper for recording results

Step 1 - Preparing a 3-5% red cell suspension
1. On the day of plasma collection, collect an additional blood sample from both donor and recipient in EDTA or serum. 2. Centrifuge the tube and separate the plasma/serum from the red cells. Elephant blood can create a gel-like plug above the clot which might need to be stirred and centrifuged to obtain an adequate serum sample. Save both. 3. Place 1 drop of recipient red cells into a small (2-5 ml) clean test tube. 4. Add approx. 1-2 ml of normal saline to the tube with the red cells. (or 1 drop RBC to 40 drops saline) 5. Centrifuge at 2500 RPM for 20 seconds. 6. Remove the supernatant, leaving the red cell button on the bottom. 7. Repeat steps 4-6 three times (for a total of 4 washes). 8. Add 1 drop of newly washed recipient red cells to a new test tube. 9. Add approximately 10-40 drops of saline and mix to suspend the red cells. This should be an approximate 3-5% washed RBC cell suspension to work with. (Haematocrit on the suspension may be performed to confirm it is 3-5%)

Step 2 - Mixing	
Minor cross match (*required for plasma donation in EEHV cases*): mix two drops of DONOR PLASMA/SERUM with one drop of washed RECIPIENT RBC suspension (optional to incubate for 15 minutes at this step), and lightly centrifuge (Approx 2500 RPM). For the control, mix one drop of RECIPIENT RBC with two drops of saline, and centrifuge.	**Major cross match**: (*required for whole blood transfusions*) mix one drop of DONOR washed RBC suspension with two drops of RECIPIENT PLASMA/SERUM, and centrifuge. For the control, mix one drop of DONOR RBC with two drops of saline, and centrifuge.
DONOR RECIPIENT 	DONOR RECIPIENT 

Step 3 - Interpretation


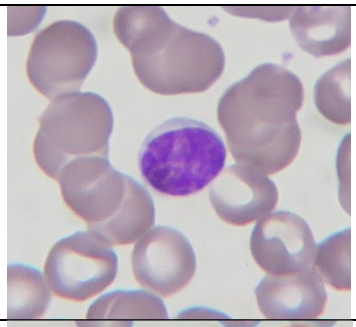
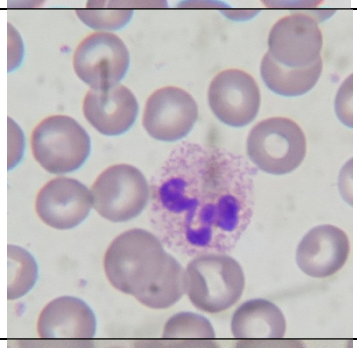
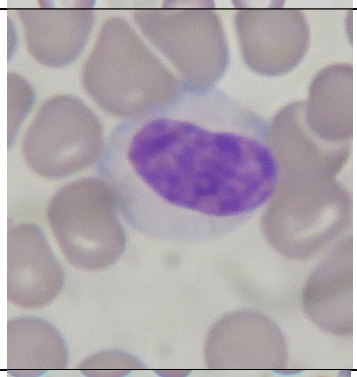
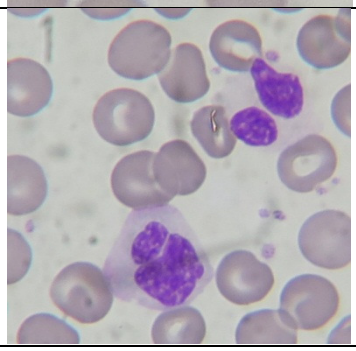
1. Check supernatant after centrifuging for signs of haemolysis. Compare with your control (control should be clear). If haemolysis is present, this cross match is incompatible. Either restart or use another donor animal.	2. Tap gently and re-suspend the cell button to see if there is any visible agglutination (macroscopic). Grade the results using the diagrams above. Compare to the control tube. Record your results.	3. Place a drop on a slide, cover with a cover slip and screen for microscopic agglutination. Record your results. This step is optional.

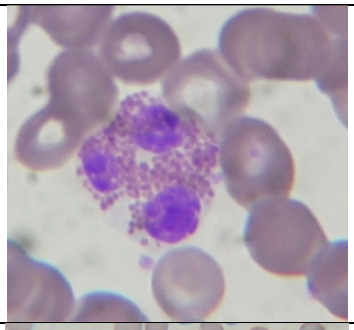
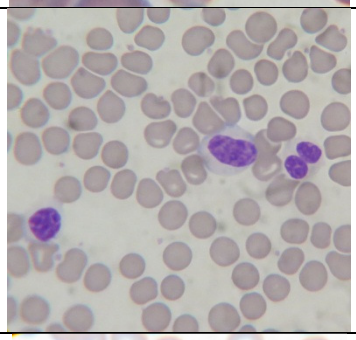
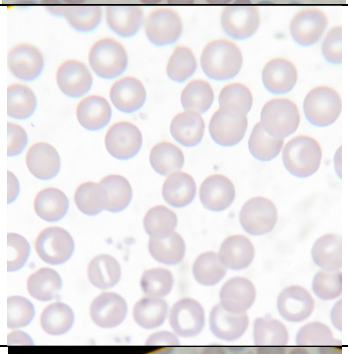
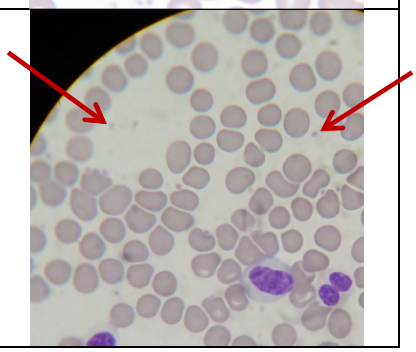
PRACTICAL ELEPHANT HAEMATOLOGY

Fieke Molenaar

Zoological Society of London, ZSL Whipsnade Zoo, Dunstable, Bedfordshire LU6 2LF, UK

Contact: fieke.molenaar@zsl.org

Lymphocyte Round nucleus Thin rim of cytoplasm, usually only around ¾ of nuclease	
Heterophil (cfr neutrophil in other mammals) Irregular, multi-lobed nucleus Irregular granules, usually eosinophilic	
Monocyte – mononuclear Large nucleus, oval to kidney shaped Cytoplasm all around nucleus Vacuoles sometimes visible in cytoplasm activated monocytes (punch out appearance) Note: elephants naturally have a high monocyte count (20-40%)	
Monocyte – binuclear Two nuclei in one large cell Cytoplasm surrounding both Most commonly found monocyte in elephants (60-80% of all monocytes)	

Eosinophil Multi-lobed nucleus Similar to heterophil Granules in cytoplasm very eosinophilic and all of them are uniformly round	
Compare lymphocyte to monocytes On the left is a lymphocyte On the right are a mononuclear monocyte and a binuclear monocyte Lymphocytes and mononuclear monocytes can look very similar and are distinguishable because monocytes have got far more cytoplasm, but this is sometimes hard to see depending on staining quality	
Red blood cells Codocytes (target cells): bulls-eye appearance Occur naturally in elephants	
Thrombocytes Can be found on each smear Stain differently with each elephant / smear quality Usually pick up some purple stain Different size and number per individual Usual numbers are 20-60 per high power field	

Workshop delegates

First name	Surname	Affiliation	Email
Adam	Kenyon	Blackpool zoo	Mammals@blackpoolzoo.org.uk
Adam	Fincher	ZSL	Adam.Fincher@zsl.org
Akbar	Dastjerdi	APHA Weybridge	Akbar.Dastjerdi@apha.gsi.gov.uk
Alanna	Straup	Independent	Alannakbell14@gmail.com
Amanda	Taylor	Veterinary student	Amandaleightaylor@yahoo.com
Andrew	Higgins	The Veterinary Journal	ajhvet@globalnet.co.uk
Andrew	Moore	Blackpool Zoo/Oakhill Veterinary Centre	andrew.moore@oakhill-vets.com
Ann	Cullinane	Dublin Zoo	jennifer.kenna@dublinzoo.ie
Anna	La Rocca	APHA Weybridge	Anna.LaRocca@apha.gsi.gov.uk
Annemarie	Kristensen	University of Copenhagen	atk@sund.ku.dk
Carol	Bradford	Albuquerque Biological Park	RShaw@cabq.gov
Chloe	Pierce	Royal Veterinary College	cpierce2@rvc.ac.uk
Christine	Molter	Houston Zoo	cmolter@houstonzoo.org
Christopher	Finnegan	APHA Weybridge	Christopher.Finnegan@apha.gsi.gov.uk
Conor	Kilgallon	Self employed	conorvet@gmail.com
Daniela	Denk	International Zoo Veterinary Group	d.denk@izvg.co.uk
Darren	Fellowes	ZSL	darren.fellowes@outlook.com
Elizabeth	Beem	Santa Barbara Zoo	lbeem@sbzoo.org
Elizabeth	Wilson	Santa Barbara Zoo	lwilson@sbzoo.org
Erica	Ward	Wildlife and Animal Welfare Institute	erica@loopabroad.com
Erin	Latimer	Smithsonian's National Zoo	latimere@si.edu
Falko	Steinbach	APHA Weybridge	Falko.Steinbach@apha.gsi.gov.uk
Fieke	Molenaar	ZSL	fieke.molenaar@zsl.org
Gareth	Richardson	ZSL	gareth.richardson@zsl.org
Gary	Hayward	Johns Hopkins University School of Medicine	gary.s.hayward@gmail.com
Hannah	Davies	APHA Weybridge	Hannah.Davies@apha.gsi.gov.uk
Isabel	Beveridge	Veterinary student	isabelbeveridge@gmail.com
Javier	Lopez	Chester Zoo	j.lopez@chesterzoo.org
Jo	Dodds	ZSL	jo.dodds@zsl.org
Joanna	Prince-Wright	APHA Weybridge	Joanna.Prince-Wright@apha.gsi.gov.uk
Joanna	Reynard	Paignton zoo	jo.reynard@paigntonzoo.org.uk
Jon	Cracknell	Conservation Medicine Services	jonwildvet@gmail.com
Jonathan	Haycock	APHA Weybridge	Jonathan.Haycock@apha.gsi.gov.uk
Karen	Archer	Oakhill Vet Centre - for Blackpool Zoo	karen.archer@oakhill-vets.com
Karla	Berry	ZSL	karla.berry@zsl.org
Katharina	Seilern-Moy	ZSL	katha_seilern@hotmail.com
Kathryn	Perrin	Copenhagen Zoo	kap@zoo.dk

Katie	Edwards	Smithsonian Conservation Biology Institute	edwardskl@si.edu
Lauren	Howard	San Diego Zoo Safari Park	LHoward@sandiegozoo.org
Lee	Sambrook	ZSL	Lee.Sambrook@zsl.org
Liam	Chenery	ZSL	Liam.Chenery@zsl.org
Linda	Van Sonsbeek	Rotterdam Zoo	l.van.sonsbeek@rotterdamzoo.nl
Louise	Guevara	Kolmården	Louise.Guevara@kolmarden.com
Mads	Bertelsen	Copenhagen Zoo	mfb@zoo.dk
Mark	Redrup	ZSL	Mark.Redrup@zsl.org
Mark	Howes	ZSL	mark.howes@zsl.org
Martina	Stevens	Houston Zoo	mstevens@houstonzoo.org
Martine	van Zijll Langhout	ARTIS Royal Zoo	m.vanzijll@artis.nl
Maurice	Lops	Copenhagen Zoo	mauricelops@hotmail.com
Maxime	Desart	University of Liège, Belgium	maxime.desart@gmail.com
Michael	Thompson	Woburn Safari Park	heidi.barrett@woburn.co.uk
Nadia	Inglese	APHA Weybridge	Nadia.Inglese@apha.gsi.gov.uk
Naomi	Spencer	Woburn Safari Park	heidi.barrett@woburn.co.uk
Nathalie	Wissink	Woburn Safari Park / Scott Veterinary Clinic	lemurcita@hotmail.com
Nic	Masters	ZSL	nic.masters@zsl.org
Pam	Lyons	Dublin Zoo	jennifer.kenna@dublinzoo.ie
Paul	Ling	Bailer college of medicine	pling@bcm.edu
Paula	Hoppe	MacArthur Balstow and Gibbs	paulahoppe@hotmail.co.uk
Peter	Bleesing	ARTIS Royal Zoo	p.bleesing@artis.nl
Petra	van den Doel	Erasmus Medical Center	p.vandendoel@erasmusmc.nl
Phillipa	Dobbs	Twycross Zoo	phillipa.dobbs@twycrosszoo.org
Priya	Bapodra	Columbus Zoo and Aquarium	priya.bapodra@columbuszoo.org
Rachel	Manchester	Royal Veterinary College	rmanchester4@rvc.ac.uk
Sarah	Chapman	UK Elephant Welfare Group	sarahhewitvet@googlemail.com
Sarah	Smith	West Midlands Safari Park	vet.nurse@wmfs.co.uk
Shane	Belson	ZSL	shane.belson@ZSL.org
Sónia	Fontes	IZW	fontes@izw-berlin.de
Sonja	Luz	Wildlife Reserves Singapore	sonja.luz@wrs.com.sg
Stefan	Groeneveld	ZSL	stefan.groeneveld@zsl.org
Stephanie	Jayson	ZSL	stephanie.jayson@zsl.org
Taina	Strike	ZSL	tai.strike@zsl.org
Tanja	Maehr	APHA Weybridge	Tanja.Maehr@apha.gsi.gov.uk
Thomas	Hildebrandt	IZW	Hildebrandt@izw-berlin.de
Viktor	Molnar	Hannover Zoo	viktor.molnar@erlebnis-zoo.de
Walid	Azab	Freie Universität Berlin	Walid.Azab@fu-berlin.de
Willem	Schaftenaar	Rotterdam Zoo	W.Schaftenaar@rotterdamzoo.nl

Workshop organisers

Dr Akbar Dastjerdi PhD
Department of Virology
APHA Weybridge, UK
Akbar.Dastjerdi@apha.gsi.gov.uk

Dr Fieke Molenaar MRCVS
Veterinary Officer
ZSL Whipsnade Zoo, UK
fieke.molenaar@zsl.org

Dr Lauren Howard DVM
Veterinarian
San Diego Zoo Safari Park, USA
LHoward@sandiegozoo.org

Lee Sambrook
Head Keeper of Elephants
Assistant Curator of Elephants
ZSL Whipsnade Zoo, UK
lee.sambrook@zsl.org

Emma Heskett
Veterinary Administrator
ZSL London Zoo, UK
emma.heskett@zsl.org