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Oceanium - Haaienzaal

First European EEHV Research Workshop

Organizing committee: ***Willem Schaftenaar**, Rotterdam Zoo*
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Program EEHV Research Workshop 20 May, 2016

	Speakers	Events
8.15		Registration
8.30	Marc Damen	Welcome by the Director of Rotterdam Zoo
8.35	Willem Schaftenaar	Introduction to the workshop
8.45	Fieke Molenaar	1. EEHV survival and fatality – screening and response protocol changes directed by clinical findings
9.00	Florence Ollivet-Courtois	2. Fatal EEHV 1B in a 8 years old Asian elephant cow and asymptomatic EEHV infection in her 1 year old brother
9.15	Kathryn Perrin	3. Thromboelastography guided diagnosis and management of a case of EEHV
9.30	Katharina von Seilern-Moy	4. Anti-herpesvirus therapy in EEHV clinical infections; a review with recommendations
9.45	Sónia Fontes	5. New approach to understand natural immunity in Asian elephants against fatal elephant endotheliotropic herpesvirus (EEHV) infection
10.00		Coffee break
10.15	Emmanuel Wiertz	6. Manipulation of the immune system by human herpesviruses: lessons for EEHV
10.30	Mathias Ackermann	7. Identification of shedders of Elephant Endotheliotropic Herpesviruses among Asian Elephants (Elephas maximus) in Switzerland
10.45	Rachael Tarlinton	8. EEHV research at Nottingham: viral excretion, cell culture, co-infections and genetics
11.00	Xander de Haan	9. Expression of the gB protein of elephant endotheliotropic herpesvirus EEHV
11.15	Petra van den Doel	10. An EEHV-gB specific capture ELISA for the detection of EEHV specific antibodies.

11.30	Taweepokee Angka-nawish	11. A serological survey for Elephant Endotheliotropic Herpes Virus among captive elephants (*Elephas maximus*) in Thailand.
11.45	Gary Hayward	12. Extraordinary levels of genetic variability amongst the envelope glycoproteins of EEHV1a strains.
12.00	Byron Martina	13. Expression of the gN protein of elephant endotheliotropic herpesvirus type 1.
12.15		Questions and answers
12.30		Lunch
13.15	Klaus Osterrieder	14. PCR protocols for the screening of elephant endoteliotropic herpesviruses types 1 to 6 Questions and answers
13.30	Alex Greenwood	15. Immunogenetics and Elephant Endotheliotropic Herpesvirus serology of captive and wild elephants and their relatives.
13.45	Paul Ling	16. Establishing assays to evaluate T cell responses to Elephant Endotheliotropic Herpesvirus (EEHV).
14.15		Coffee break
14.30		General discussion, priority list
(15.00		Coffee break)
15.05	Byron Martina	Priority topic 1: DIAGNOSTICS
(15.45		Coffee break)
15.50	Paul Ling	Priority topic 2: EPIDEMIOLOGY
(16.30		Coffee break)
16.35	Alex Greenwood	Priority topic 3: VACCINE AND TREATMENT
(17.15		Coffee break)
17.20		Conclusions, closing.
17.45		Aperitif
18.30		Dinner

Abstracts

1. EEHV survival and fatality – screening and response protocol changes directed by clinical findings

Fieke Molenaar[1], Nic Masters[1], Katharina Seilern-Moy[2] and Akbar Dastjerdi[2]

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ZSL Whipsnade Zoo has seen its fair number of Asian elephant calf fatalities: Emelia in 2006 (EEHV1b), Riddle and Bets in 2009 and Max most recently in 2015 (all EEHV1a). There has also been one survivor: in 2013 Scott temporarily showed clinical signs coinciding with a high viral load of EEHV1a.

Increased monitoring of the breeding herd at ZSL Whipsnade Zoo, combined with extensive sample collection, especially of the last two clinical cases, has contributed to a better understanding of the clinical progression of the disease, which has led to changes in the screening and response protocols. Main findings were the presence of viraemia well before the onset of clinical signs, the potential influence of minor clinical issues (in one case a temporal gland infection) on triggering rapid viral replication, the importance of thrombocyte counts as a prognostic factor throughout the (subclinical) disease process, and the uncertainty of the efficacy of rectal famciclovir, especially when the intestinal circulation becomes compromised.

The new screening protocol at ZSL Whipsnade Zoo consists of weekly full blood sampling of all juveniles less than eight years of age for PCR analysis and in-house white blood cell differentials and thrombocyte counts. Individuals that show a single high DNAemia on PCR, or an increase in DNAemia or a significant decrease of thrombocytes on two consecutive samples will be treated. This will offer a larger window of opportunity to start treatment at the early stages of viral replication, before extensive vascular damage.

First line treatment will consist of intravenous and rectal fluids to ensure adequate circulating volume, intravenous plasma from suitable donors to provide antibodies and thrombocytes, intravenous ganciclovir instead of oral or rectal famciclovir to attempt to slow down viral replication and parenteral antibiosis to combat the suspected disseminated intravascular coagulation that could be the cause of the progressive thrombocytopenia. Throughout this time, the viral load will be closely monitored to ensure efficacy of anti-herpes viral treatment. All juveniles at ZSL Whipsnade Zoo have been trained to

accept blood sampling, rectal fluids and placement of intravenous cannulas without the need for sedation, to minimize additional stress caused by unfamiliar intensive handling that may exacerbate the infection outcome.

2. **Fatal EEHV 1B in a 8 years old Asian elephant cow and asymptomatic EEHV infection in her 1 year old brother**

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Jade is an 8 years old Asian elephant cow. She presented signs of weakness, slight leucopenia and thrombocytopenia, low albumin and total proteins the day before her death with no oral lesion or tongue petechia before her death. Keepers mentioned slight pallor of the mucous membranes. Necropsy was highly compatible with EEHV infection with multiple hemorrhages and petechia including in the tongue. Histology revealed marked and acute multicentric congestion and hemorrhages in heart, fat tissues, lymph nodes, spleen, liver, lungs, kidneys with endothelial intranuclear inclusions and moderate to marked chronic nephritis. Quantitative PCR confirmed EEHV1b infection with negative ELISA antibody test.
All 4 adults of the herd were checked daily since then and presented no oral lesions at any time. Quantitative PCR done every week, were negative in all adults except in Nina (mother of Jade), 10 days after Jade death and Accra 36 days after. Their PCR were negative the week after. The bull “Max” has ELISA positive result in serum banked the year before.
Tom, 1 year old male, presented petechia latero-ventrally on his tongue during 1 week with no general illness. Six days after the death of Jade the team was able to do the first blood sample by training. Hematocrit was low with leukocytosis. Quantitative PCR was highly positive and remained notably positive 49 days. Quantitative PCR became fully negative 1 month later. Famciclovir treatment started the first day of the EEHV episode with 10mg/kg TID. Coconut oil and coco nuts were added to the diet for the protective potential this fruit might present. Famciclovir treatment was reduced to 10mg/kg BID, 20 days later and stopped when q PCR became very low 49 days later. No side effects could be noticed despite the long treatment.

These cases of EEHV infection are uncommon because an 8 years old elephant died which is quite old and because EEHV 1b is involved which is

less common than EEHV1a. Early training of babies for blood samples and proper oral checking is essential. All breeding facilities must be prepared to face EEHV infection with ready action plan (drugs, labs contacts, checklist of samples…). All institution should have individual normal values for each healthy elephant because ISIS min and max values are not always relevant. Good coordination and agreement of all labs are essential to make progress and save animals.

3. Thromboelastography and its applications to EEHV cases

Kathryn L. Perrin,[1] Annemarie T. Kristensen,[2] Dennis Schmitt,[3] Wendy K. Kiso,[3] Lauren Howard[4] and Mads F. Bertelsen.[1]

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Thromboelastography (TEG) is a functional test of coagulation which can be performed on whole blood and, as such, is a much more sensitive indicator of coagulopathies than conventional plasma based tests.[1] However, when applying this test to the monitoring of juvenile elephants at risk of EEHV-Haemorrhagic Disease (EEHV-HD), there are uncertainties in terms of access to TEG machinery and basic knowledge necessary for interpretation.
TEG was used to demonstrate severe hypocoagulability in a case of EEHV-HD in 2014.[2] Modest improvements were seen in TEG parameters after multimodal therapy, including plasma and human recombinant factor VII. TEG may be useful in both routine monitoring of calves, as well as during viraemia episodes, to help inform clinical decision making.
Currently we are establishing reference intervals in healthy elephants. Citrated whole blood samples were analyzed after 60 minutes (fresh) and 24 hours (refrigerated) to investigate the feasibility of shipping samples to an external laboratory. Additionally frozen plasma samples will be analyzed shortly, to investigate the use of TEG on archived samples. Lastly, samples are being collected to document the haemostatic changes during viraemia episodes in juvenile animals. This is part of a wider project to document the host response to EEHV infection.
References
1. Wiinberg, B. & Kristensen, A. T. Thromboelastography in veterinary medicine. *Semin. Thromb. Hemost.* 36, 747–56 (2010).

2. Perrin, K. L., Kristensen, A. T., Krogh, A. K. H., Grøndahl, C. & Bertelsen, M. F. Thromboelastography-guided diagnosis and therapy in case of elephant endotheliotropic herpevirus hemorrhagic disease. in *Proceedings of the Annual Conference of the American Association of Zoo Veterinarians* 84–85 (2015).
Acknowledgments: We would like to thank Peter Fischer for providing us with equipment and technical assistance and Houston Zoo, Ringling Center for Elephant Conservation and Woburn Safari Park for providing blood samples. Also thanks to Louise Bochsen and Anne Krogh for their technical help and training.

4. Anti-herpesvirus therapy in EEHV clinical infections; a review with recommendations

Katharina Seilern-Moy, MedVet,[1,2] Fieke Molenaar, DVM, MSc, GPCert(ExAP), MRCVS[3], and Akbar Dastjerdi, DVM, MSc, PhD[1]

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The majority of fatalities in juvenile Asian elephants are attributed to the clinical infection with Elephant Endotheliotropic Herpesviruses (EEHVs). The viruses' role in these fatalities is known since the 1980s, but effective control of the clinical infections faces numerous challenges. Recent achievements in decoding the viruses' genome (Ehlers at al., 2006; Wilkie et al., 2013) have revealed genes with products, such as thymidine and protein kinases, essential to process anti-herpesvirus prodrugs to their active metabolites. This along with better understanding of the viruses' dynamics of infection (Stanton et al., 2013) are gradually advancing our knowledge to devise more effective disease management protocols.

The anti-herpesviral drug famciclovir (FCV), used to treat human infections with alpha herpesviruses such as the herpes simplex virus, has traditionally been used to treat EEHV viraemia in at-risk elephants. Recently, aciclovir (ACV) and ganciclovir (GCV), two first line drugs for treatment of alpha and beta herpesviruses' infections in humans, have also been administered to viraemic elephants. There are reports of several elephant calves that have survived viraemia or clinical EEHV-1 infections whilst being treated with the anti-herpesvirus drugs. On the other hand, many animals have also died despite receiving the antiviral medications. It is reasonable to believe that a selection of the viraemic survivors might have overcome the infection even in

the absence of the anti-viral drug therapy. This presentation briefly describes the modes of action of anti-herpesviral drugs and their preference applications in human treatment of herpesviruses. Further, it discusses clinical cases of viraemic elephants with respect to the viral load and anti-viral drug used with the aim to highlight alternative treatment options for potentially fatal viraemia in at-risk elephants.

REFERENCES

- Ehlers B, Dural G, Marschall M, Schregel V, Goltz M, Hentschke J. 2006. Endotheliotropic elephant herpesvirus, the first betaherpesvirus with a thymidine kinase gene. J. Gen. Virol. 87:2781–2789
- Stanton JJ, Zong JC, Eng C, Howard L, Flanagan J, Stevens M, et al. 2013. Kinetics of viral loads and genotypic analysis of elephant Endotheliotropic herpesvirus-1 infection in captive Asian elephants (Elephas maximus). J Zoo Wildlife Med. 44(1):42–54
- Wilkie GS, Davidson AJ, Watson M, Kerr K, Sanderson s, Bouts T, Steinbach F, Dastjerdi A. 2013. Complete genome sequences of elephant endotheliotropic herpesviruses 1A and 1B determined directly from fatal cases. J. Virol. 87:6700–6712

5. **New approach to understand natural immunity in Asian elephants against fatal elephant Endotheliotropic Herpesvirus (EEHV) infection**

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Since the first reported case in 1990, Elephant Endotheliotropic Herpesvirus (EEHV) has claimed the life of nearly one third of all Asian elephant calves captive born in Europe.
In North America and Europe, there are breeding facilities which are highly affected by EEHV hemorrhagic disease with up to 100 per cent of calf mortality and others which are not, or just minimally, affected by this disease. Hand-reared calves are at a higher risk of EEHV infection, and also the weaning period represents a very critical phase for a young calf regarding this infection. Therefore the Asian elephant milk seems to contain one or more protective component(s), which are responsible for safeguarding the calves during active lactation. To explain the high risk of infection under Zoo conditions one or more protective elements in the life of young elephants must

be assumed to exist in their natural habitat, which allowed Asian elephants to co-exist with EEHV over the last 4 million years.
The aim of this project is to recognize the most important predictive elements in the individual life history of calves (until the age of 3 years), which allow them to resist or which make them especially sensitive to EEHV. It embraces also the investigation of the protective compounds existing in the wild, and targets for a better understanding if an involvement of a coagulation disorder can be present in the onset of this hemorrhagic disease.

6. Manipulation of the immune system by human herpesviruses: lessons for EEHV

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In the course of millions of years of co-evolution with their hosts, herpesviruses have acquired a wealth of strategies to manipulate the host immune system. Dedicated immune evasion molecules allow herpesviruses to effectively elude both innate and adaptive immunity. These immune evasion mechanisms facilitate the establishment of lifelong infections. The virus occurs as a latent infection from which it reactivates occasionally to generate new infectious particles. Examples of herpesvirus immune evasion strategies and their role in the viral life cycle will be presented for alpha-, beta- and gammaherpesviruses.
If primary infection occurs at an early age, this is usually asymptomatic; however, if an individual is first infected at a later age, this often results in disease. For example, primary infection of children with Epstein-Barr virus (EBV) before two years of age is mostly asymptomatic; however, primary EBV infection in adolescents results in mononucleosis in up to 70% of the cases. Mononucleosis is related to a strong immune response against EBV. The age at which primary infection with EBV occurs correlates with the socio-economic status of the population.
The situation in young elephants infected with EEHV may resemble the clinical pattern of mononucleosis in humans. If primary infection of elephant calves occurs during the first year, the animals may be protected against disease by maternal immunity. Also, the immune system may respond mildly against the virus at this young age. Yet unknown viral evasion mechanisms are likely to facilitate the establishment of a latent EEHV infection. However, if primary infection occurs at a later age, protection by maternal immunity will

no longer be effective, whereas the mature immune system may respond strongly against the virus, causing serious morbidity and occasionally even mortality.
Primary infection of elephant calves may be delayed in small, isolated populations due to limited circulation of infectious virus. These conditions may explain the typical epidemiology observed for EEHV.
Suggestions for clinical management of EEHV infection and prevention of EEHV-associated disease will be discussed.

7. **Identification of shedders of Elephant Endotheliotropic Herpesviruses among Asian Elephants (*Elephas maximus*) in Switzerland**

Mathias Ackermann[1], Jean-Michel Hatt[2], Nelli Schetle[1], Hanspeter Steinmetz[3]

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Abstract to be distributed at a later stage.

8. **EEHV research at Nottingham: viral excretion, cell culture, co-infections and genetics**

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Work on EEHV at Nottingham University and Twycross zoo has focussed on the epidemiology of the virus in the elephant population. We have published work on the shedding of the virus in one herd during several pregnancies that indicates that viral shedding is more likely related to behavioural stressors than pregnancy per se. We have also successfully cultured endothelial cells from elephant umbilical cords (though not virus). We have identified the virus in trunk wash material using next generation sequencing technology and screened DNA/RNA from tissues from EEHV clinical cases using a microarray screen and qPCR for rodent borne pathogens including EMCV (with negative results for any viruses other than EEHV) . We have also

performed some preliminary work examining potential familial links with EEHV clinical cases which we will present.

9. Expression of the gB protein of elephant endotheliotropic herpesvirus EEHV

Jojanneke Dekkers[1], Susan Fekken[2], Ab Osterhaus[2], Frank van Kuppeveld[1], Petra van den Doel[3], Byron Martina[2], C.A.M. (Xander) de Haan[1]

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Viral attachment and fusion proteins are main targets of the host immune system. Recombinant versions of these proteins can be used to detect antibodies in sera or as vaccine antigens. The Virology Division of the Faculty of Veterinary Medicine of the Utrecht University has ample experience with the expression of recombinant fusion proteins of influenza, corona- and respiratory syncytial viruses. Eukaryotic expression systems are used for the expression of these glycoproteins as glycosylation plays an essential role in their proper folding and oligomerization. In the present study we aimed to express the gB protein of genotype 1A EEHV, either as recombinant soluble or as membrane-anchored protein. Just as other fusion proteins, gB is a metastable protein that may refold from the prefusion into the postfusion conformation. To promote expression of the prefusion form, the gB ectodomain was mutated at its putative fusion peptides and at the furin cleavage site. In addition, the ectodomains were fused either to different oligomerization domains or to a transmembrane domain. For some constructs, low levels of soluble Gb proteins were expressed that were secreted from cells. These proteins may be used for the development of a capture ELISA to detect Gb-specific antibodies in sera. In addition, transmembrane containing Gb proteins could be easily detected in transfected cells by using rabbit sera induced by gB-specific peptides or different elephant sera. This latter construct can be used for the detection of gB-specific antibodies for example by using an immunofluorescence assay and for the development of heterologous vaccine vectors that express gB. gB proteins of other EEHV genotypes probably can be expressed using a similar approach.

10. A Novel Antigen Capture ELISA for the Specific Detection of IgG Antibodies to Elephant Endotheliotropic Herpes Virus

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Until recently EEHV could only be detected by PCR techniques in animals with an active and shedding infection, whereas latent EEHV carriers can only be detected with a serological assay. The development of a serological assay has been a major challenge since the EEHV virus could not be grown in cell culture and recombinant expression of the EEHV capsid proteins was unsuccessful.
An EEHV specific capture ELISA was developed in 2013 based on EEHVgB-1A expressed in *E.coli* with a C-terminal histag. This antigen was expressed full length (87 kD) as well as several smaller fragments of different molecular weights. The antigen was NiNTA purified however still contained a considerable portion of *E.coli* contaminants which were reactive with rabbit as well as elephant sera. To exclude aspecific *E.coli* reactivity, the antigen was captured by a histag antibody and the assay could be further developed to an EEHV specific serological assay. However, a high background signal, which could not be reduced, makes the test less sensitive and truly negative sera could not be discriminated from those with very low titres.
This assay was applied on large cohorts of Asian elephant sera in Europe, USA and Thailand. A vast portion of the Asian elephants displayed antibodies against EEHV underlining the fact that elephants are the natural host of EEHV. In 80% of the EEHV PCR positive elephants antibodies were detected. Even in those elephants which were diagnosed with other EEHV subtypes than EEHV-1A.
The monitoring of three European herds over a long period of time (250 up to 600 days) showed that within a herd the antibody levels per animal may vary from non-detectable up to high titres. Those who display antibodies show herpesvirus-like rise and fall patterns over time and other seropositive herd members peak more or less simultaneously. Moreover, we found elephants with continuous high titres. Most of them were genetically related to EEHV fatal cases (siblings, cousins); relationship analysis and epidemiology are warranted to test whether this hypothesis holds true.
Despite the results of these large cohorts, no significant conclusions could be drawn on the EEHV fatal disease. Our test results should be confirmed with an

improved serological assay and better design of the study cohorts, which include serology as well as PCR techniques, is warranted in order to perform epidemiological studies.

11. A serological survey on Elephant Endotheliotropic Herpes Virus among captive elephants (*Elephas maximus*) in Thailand.

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In the last two decades, the Elephant Endotheliotropic Herpesvirus (EEHV) has been the main cause of death in young captive Asian elephants (*Elephas maximus*) (Garner et al, 2009; Long et al, 2015). EEHV is a β-herpesvirus associated with elephants. Elephants in the age of 1-10 years, may die within 1-3 days after showing the first clinical signs of infection due to heart and/or (multiple) organ failure, caused by vascular damage. Long et al (2015) estimated that around 20 % of Asian elephant calves are susceptible to primary infection with fatality rates as high as 85%. Survivors, like young and adult elephants infected with or without apparent diseases, become latently infected and may shed virus via secretions. In Thailand thirty-one elephants have been diagnosed as EEHV infected using PCR between 2006 and 2015 (EEHV task force, Thailand 2016).

The present study was the first performed in Thailand to assess EEHV specific antibodies by an ELISA test using recombinant EEHV1A-glycoprotein B (*gB*) peptides as antigen (van den Doel et al, 2015). Longitudinal serum sampling of 47 elephants, housed in the National Elephant Institute (NEI) facilities in Lampang, was done during the period 2007-2014. Antibody titre profiles in course of time will be reported. In this group one infected survivor elephant revealed EEHV-1A infection by PCR during severe clinical illness. In a large cross sectional survey antibody levels, expressed in Optical density (OD) units, were determined in elephants (n= 994) throughout Thailand, and the

correlation between OD levels and (risk) factors potentially related with EEHV infection, like elephant age, gender, location of camp in the geographic regions North, North-East, East, West, Central and South was investigated. Camps in each region were grouped based on clustering with other camps and elephant numbers in each cluster were estimated. The EEHV seroprevalence in this cross sectional survey and preliminary data concerning infection associated factors will be presented.
It is our preliminary conclusion that in view of a seroprevalence of 26 % (263/994) it is highly likely that elephants infected with EEHV with or without associated disease symptoms, are present throughout one fourth of the total captive elephant population (n= 4,016) in the country, and have the potency to transmit the virus, maintain it in the population and potentially spread it into the wild elephant population as well. This urges for additional research concerning the immunopathogenesis of EEHV infections and potential genetic factors involved in survivors.

12. Extraordinary levels of genetic variability amongst the envelope glycoproteins of EEHV1a strains.

Zong, J-C, Long SY, Heaggans SY, Zachariah A, Pearson VR, Latimer EM and Hayward GS

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All four major EEHV genome types that have been completely sequenced (EEHV1A, EEHV1B, EEHV5 and EEHV4) retain six core glycoproteins gB, gH, gM, gN, gO and gL in common with all other mammalian herpesviruses. The bulk of the novel segments of these genomes also encode varying complements of an ancient tandemly-duplicated gene family consisting of some 25 distantly related paralogous members of 7X transmembrane proteins, including a large subset with features that resemble orphan chemokine-receptors (vGPCRs). However, in addition they also encode another set of Proboscivirus genus and species-specific spliced anchored N- and O-linked envelope glycoproteins known as ORF-O, P, Q and R, as well as multiple immunoglobulin-like family proteins that all display extraordinary diversity and heterogeneity both between species and subtypes as well as amongst different individual strains of even the most highly pathogenic type EEHV1A itself. We have known for many years that almost all individual EEHV1A

strains found at different facilities worldwide display high levels of genetic polymorphisms. This occurs at such high levels that all epidemiologically distinct strains are easily discriminated by DNA sequence data (PCR gene subtyping) at selected variable gene loci (such as vGPCR1 and the three vOX2 family proteins) across the 180-kb genomes. The results can then be used to generate "phylogenetic family trees" at each characterized PCR locus amongst the more than 60 different strains of EEHV1A identified so far from both lethal and surviving HD cases or from asymptomatic shedding in Asian elephants.

More recently, as will be described here, we have also now evaluated levels of protein divergence for more than 30 EEHV1 strains at three other truly amazingly diverged segments encompassing nearly 10% of the intact EEHV1 genomes. These are, in order of increasing complexity, firstly, the 2.2-kb glycoprotein-H gene region within CD-II; secondly, the 4.1-kb combined ORF-O, P and Q region within CD-III, and thirdly, a very complex 10-kb block at one end of the genome encompassing different numbers and arrangements of three distinct paralogous membrane-anchored gene families, including a set of diverged CD148-like proteins. At the first locus, the entire conserved 700-aa gH protein amongst EEHV1A strains falls into five major largely invariant sub-type clusters that differ by between 7 and 15% and are diverged from the EEHV6 versions by 20% and from the EEHV1B versions by 35%. At the second locus, the EEHV1A and EEHV1B versions of all three proteins are even further diverged and both ORF-P and ORF-Q fall into at least three to four major subtypes amongst even just the EEHV1A strains (with 40 to 60% amino acid differences). Finally, the third region is so complex in terms of different contents of intact versus fragmented genes and numbers of paralogous family members that there is no residual match whatever to the previously described and otherwise linked EEHV1A versus EEHV1B subtype CD-I/II/III patterns. Evidently the extant EEHV1 viruses are a very ancient mixed population with far greater levels of scrambled chimerism than previously recognized. Based on the precedents from other mammalian herpesvirus branches, many of these group-specific hypervariable membrane glycoproteins are likely to have major roles and influences on host-cell receptor interactions and on neutralizing antibody responses. Therefore, we expect that there will likely be extraordinarily high levels of functional heterogeneity displayed not just between the seven different EEHV species themselves, but also amongst and between individual EEHV1A strains.

13. Expression of the gN protein of elephant endotheliotropic herpesvirus type 1

Stephanie Lim, Loubiela Joseph, Jeroen Roose, Ab Osterhaus, Byron Martina

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At least twelve glycoproteins have been identified in some herpesviruses, which are involved in cell binding and entry, cell-to-cell spread, and egress. Although glycoproteins B and D are the most immunogenic ones, vaccine candidates against the other glycoproteins have also been shown to be effective. The gM/gN is of particular interest. Despite its nonessential status in tissue culture, gM of several herpesviruses has been associated with a number of functions throughout the viral life cycle. The glycoprotein is known to down-regulate the surface expression of gD and the gH/gL complex, two key players in virus-induced membrane fusion, and facilitates the upstream incorporation of the gH/gL complex into mature virions. Furthermore, gM has been shown to stimulate viral entry in the context of syncytial strains. However, despite its presence on nuclear membranes gM is seemingly not involved in the release of herpes-viruses from the nucleus, where newly made viral capsids are initially assembled. In contrast, with the conserved gN viral protein, gM alters immunity against the virus by down-regulating the transport and peptide loading of major histocompatibility complex class I in the endoplasmic reticulum (ER). Finally, and perhaps most interestingly, gM has been reported to modulate virulence in animal models. Thus, gM appears to exert important and diverse regulatory activities at potentially different intracellular localizations. The exact mechanism of action of gN is still unknown, but it may modulate gM by potentially sequestering it and/or preventing it from interacting with other proteins. Some studies have also shown that during natural infection with herpesviruses antibodies are generated against gN. Therefore, we started studies to investigate the suitability of gN for development of a serological assay and the combination gN/gM as a possible vaccine candidate. To this end, gN was cloned using the Leishmania system. The preliminary data indicate that protein is made, although not in a secreted form. Furthermore, it is possible to develop an ELISA system to monitor the immune response of elephants. Studies are ongoing to monitor the immune status in selected elephant herds and develop an MVA-based gM/gN candidate vaccine

14. PCR protocols for the screening of elephant endoteliotropic herpesviruses types 1 to 6

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PCR-based protocols are used in the laboratory for the screening of elephant endotheliotropic herpesviruses type 1 to 6. First, a sensitive probe based Real time PCR for the detection of EEHV-1 described by Hardman et al. (2012) was optimized using DNA extracted from organs of a positive EEHV-1 case. For a rapid screening, a single PanPol Sybr Green protocol (primers from Latimer et al., 2011) for EEHV-1, -2, -5 and 6 using synthetic DNA as positive controls is in use in the laboratory. Digestion of the amplified product or specific probe-based assays can be used as a confirmatory tool. EEHV-3/4 are screened using a probe-based PCR targeting the same POL gene region.

15. Immunogenetics and elephant endotheliotropic herpesvirus serology of captive and wild elephants and their relatives

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Pathogens and host co-evolution can shape the genetic diversity of animal populations, particularly the immunogenetic loci. This dynamic has been intensively studied over both the short term within currently existing populations and over the long term by comparing different species where much of the intervening development of immunogenetic diversity can only be inferred. The association between genetic diversity, specific immunogenetic polymorphisms and health is a very active area of research and is yielding many insights into host adaptations to pathogens. We are focusing our efforts on the study of genetic diversity within the major histocompatibility loci (MHC) and the pathogen sensing toll-like receptors (TLRs) in elephants, woolly mammoths and both ancient and modern muskoxen as part of a Deutsche Forschungsgemeinschaft (DFG) funded project. We hope to

understand the dynamics of the evolution of these genes over the long term. We are also interested in comparing the immunogenetic diversity of captive versus wild elephants. The goals of this aspect of our work is to examine whether the frequency distribution of specific alleles in the immune related genes are different in the captive elephant population as compared to the wild and whether or not specific immunogenetic polymorphisms correlate with elephant endotheliotropic herpes (EEHV) related disease in captivity. We are also investigating the potential use of EEHV specific peptides to develop viral strain specific serological tests to examine captive and wild elephants for strain specific exposure to EEHV and EEHV co-infection history. Taken together we are exploring whether immunological differences can explain or correlate with EEHV disease progression. The projects have approval from the Elephant TAG.

16. Establishing assays to evaluate T cell responses to Elephant Endotheliotropic Herpesvirus (EEHV)

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Elephant Endotheliotropic Herpesvirus (EEHV) can cause lethal hemorrhagic disease in both Asian and African elephants, especially in juveniles. Despite advances in EEHV research, there remains a gap in our understanding of immunological responses to EEHV, particularly T cell responses. To address this issue, we generated or identified antibodies specific to elephant surface markers and cytokines to establish ELISpot and flow cytometry-based assays to detect antigen specific responses. To evaluate the sensitivity and robustness of these first generation assays, we first tested T cell responsiveness by taking advantage of rabies, which captive elephants are routinely vaccinated against. We detected strong IFN-γ responses to rabies nucleo-capsid 2-3 weeks following vaccination, which were confirmed, via flow cytometry, to be predominantly from CD8+ T cells. We also detected IFN-γ responses following long term expansion cultures, which will likely be needed to detect low frequency EEHV positive T cells in normal healthy but previously infected animals. Ongoing studies are using these newly developed assays to screen EEHV proteins, initially focusing on predicted regulatory or structural proteins, which have been found to induce potent T cell responses following infection with other herpesviruses.

Workshop Program

19 May 2016: **Clinicians meeting, 16:00**

Location: Veterinary clinic, Rotterdam Zoo

Welcome drinks, 08:00 PM

Location: Van der Valk Hotel Rotterdam-Blijdorp (lobby), Energieweg 2, 3041 JC Rotterdam

20 May 2016 **Workshop, 08:00 AM – 05:45 PM**

Location: Haaienzaal, OCEANIUM of Rotterdam Zoo

Aperitif: 05:45 PM – 06:30 PM

Location: Caribbean Café (OCEANIUM)

Dinner: 06:30 PM – 08:00 PM

Location: Haaienzaal (OCEANIUM)

Please use the main entrance of the zoo: Blijdorplaan 8, 3041 JG Rotterdam

Free entrance to the zoo by showing the cover of the program at the gate.

The gate opens at 8:00 AM.