Case Report

Fatal elephant endotheliotropic herpesvirus-1 and -4 co-infection in a juvenile Asian elephant in Europe

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Introduction
Elephant endotheliotropic herpesviruses (EEHVs; also known as elephantid herpesviruses), in the genus Proboscivirus, are one of the leading causes of mortality in juvenile Asian elephants (Elephas maximus). The viruses are considered as a significant threat to conservation of Asian elephant populations and are now thought to be ubiquitous in Asian elephant populations (Reid et al., 2008; Hardman et al., 2012; van den Doel et al., 2015). EEHV-1 has been responsible for the majority of herpes-associated fatalities in Asian elephants worldwide. On occasions, other EEHV genotypes, i.e. EEHV-3 (Garner et al., 2009), -4 (Sripiboon et al., 2013) and -5 (Denk et al., 2012), have also been reported as the cause of Asian elephant deaths in the USA, Asia and Europe, respectively. In this case report we describe the investigation into a juvenile Asian elephant fatality in a European zoo.

Case Presentation
A fatal case of haemorrhagic disease in a juvenile Asian elephant from a European zoo was diagnosed with co-infection of EEHV-1 and -4. EEHV-4 had a wider organ distribution and a higher viral load; both viruses presented the highest load in the mesenteric lymph nodes.

Conclusion
Detection of EEHV-4 in this fatal case in Europe underlines the importance of inclusion of all known Asian EEHVs in routine blood monitoring to facilitate early therapeutic intervention.

Keywords: Asian elephant; co-infection; elephant endotheliotropic herpesvirus; Europe; haemorrhagic disease; multiple haemorrhages.

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Case report
A fatal case of EEHV-1A infection in a 2-year-old male Asian elephant at Copenhagen Zoo in November 2014 led to renewed investigation into a previous fatality in 2003. This case, another 2-year-old male Asian elephant, housed at the zoo along with six other Asian elephants, showed signs of mild depression and lethargy on 4 October 2003. The animal was not tractable and a cursory clinical examination did not reveal any significant abnormalities. On day 3, the animal was seen alive on surveillance cameras at 06.48 a.m., but found dead at 07.15 a.m.

Investigations
At necropsy, pathological findings revealed severe vascular damage reminiscent of that described previously for fatal...
cases of EEHV infections (Garner et al., 2009; Wilkie et al., 2014). The most significant gross pathological findings included 5–10 l of serohaemorrhagic fluid in the abdomen, diffuse petechial haemorrhages and extensive oedema of all serosal surfaces, multiple haemorrhages in the heart and liver as well as well-defined haemorrhagic lesions throughout the renal papillae and adrenal cortex (Fig. 1). The small intestine contained fluid ingesta intermixed with blood and extensive punctuate ulcerations (5–20 mm diameter). The ulcers, primarily co-localizing with the Peyer’s patches, were present particularly throughout the aboral portion of the small intestinal mucosa. The mucosa of the large intestine was oedematous, dull and showed generalized dark reddening.

Histopathological examination of the tissues confirmed widespread haemorrhages, and severe small intestinal and colonic submucosal oedema. In the small intestine, there was mild-to-moderate submucosal haemorrhage and within the small intestinal crypts, mitotic activity was judged to be very low (less than one mitotic figure per 10 crypts). Peyer’s patches were reduced in size and showed central necrosis of the peripheral follicles as well as marked interstitial haemorrhage. The overlying epithelium was intact, but had collapsed into the space created by the reduced size of the Peyer’s patches. In the colon, mild heterophilic infiltrates were seen in the lamina propria, associated with moderate-to-severe mucosal haemorrhage.

The endothelial cells of the large myocardial arteries and the endocardium had remarkably high numbers of single large basophilic intranuclear inclusion bodies (up to 20 per high-power field) (Fig. 2). Similarly appearing, but few and scattered inclusions were also present in arteries of the lung, liver, kidney, urinary bladder and skeletal muscle. Mild endarteritis with sloughing of endothelial cells and cellular infiltrates consisting of lymphocytes and

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**Fig. 1.** Gross pathological findings in the EEHV-1 and -4 co-infection fatality. (a) Mucosa of ileum showing necrotic lesions (5–20 mm diameter) and bloody intestinal contents. (b) Mucosa of the large intestine showing oedema and haemorrhage. (c) Kidney showing marked haemorrhages in the renal papillae. (d) Adrenal gland showing severe cortical haemorrhages.
heterophils, some of which were necrotic, was also seen in the same locations. Bacterial culture of the intestinal mucosa, faeces and major organs was unremarkable. DNA from the various organ samples, stored at 220°C until processing, was extracted using an EZ1 RNA Tissue Mini kit on an EZ1 XL robot (Qiagen) and tested for EEHV-1, -3, -4 and -5 using simplex real-time quantitative PCR (qPCR) assays (Hardman et al., 2012; unpublished data). DNA sequencing of conventional PCR amplicons derived from lung and heart tissue samples was carried out using an ABI 3130xL 16 capillary genetic analyser (Applied Biosystems). SeqMan (DNASTAR) was used to analyse sequence traces and the consensus sequences were screened against GenBank entries using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The EEHV DNA was prepared for next-generation sequencing libraries using a Nextera XT kit (Illumina). Paired-end sequencing was performed on an Illumina MiSeq. The sequences were then mapped to the reference EEHV-1A Raman strain (GenBank accession number KC462165.1) and several EEHV-4 sequences in GenBank using SeqMan NGen software (DNASTAR). Several conventional PCRs were also used to generate DNA standards for EEHV-1A and -4 to quantify the viral loads in the tissues. The genome copies for EEHV-1A and -4 from each tissue were then normalized to 1 μg extracted total nucleic acid.

**Diagnosis**

The viral genotypes identified in the organs by qPCR and DNA sequencing were EEHV-1A and -4. The BLAST search of 201 nt of the EEHV-1A PCR amplicon (GenBank accession number KT381607) revealed the highest similarity (100 %) to the DNA polymerase gene (U38) of reference EEHV-1A Raman strain and several other EEHV-1A strains in GenBank. Sequences of other shorter genome segments, corresponding to nt 29 539–29 638 (U73, DNA replication origin-binding helicase), 31 935–32 053 (U72, envelope glycoprotein M) and 50 617–50 750 (U56, capsid triplex subunit 2) on the Raman strain genome, produced similar results in the BLAST search. Nucleotide sequences of the EEHV-4 DNA polymerase (U38, 301 bp), glycoprotein M (U72, 303 bp) and DNA replication origin-binding helicase (U73, 262 bp) gene segments (GenBank accession numbers KT334336–KT3343368) were identical to corresponding sequences of EEHV-4 strains from North America (GenBank accession numbers JN983096.1, EU658934 and JN983097.2). Using qPCR, EEHV-1A DNA was only detectable in the heart, mesenteric lymph node, lung and spleen, whereas EEHV-4 DNA was detectable in all organ samples tested (adrenal gland, heart, ileum, mesenteric and pulmonary lymph nodes, lung, spleen, testicle and thymus) (Table 1). The highest number of EEHV-4 viral genome copies, normalized to 1 μg total nucleic acid, was found in the mesenteric lymph node (3.38 × 10^8 copies μg^-1), followed by the adrenal gland, lung, spleen and heart (Table 1). EEHV-1A virion loads appeared to be far lower than those of EEHV-4. The highest viral load was detected in the mesenteric lymph node (2.02 × 10^4 copies μg^-1), followed by heart and lung; however, the differences in viral load of lymph node and heart were negligible (2.02 × 10^4 versus 1.22 × 10^4 copies μg^-1). Irrespective of the tissues tested, the viral load for EEHV-4 was always higher, between 1.3 and 16 600 times, than those of EEHV-1A. The EEHV-1A load was also on average between 42 and 6600 times lower than that typically seen in corresponding organs of other EEHV-1 fatalities (unpublished data). However, whether

**Table 1. EEHV-1A and -4 mean viral genome copies in various tissues of a juvenile Asian elephant using qPCR**

<table>
<thead>
<tr>
<th>Virus type</th>
<th>Adrenal</th>
<th>Heart</th>
<th>Ileum</th>
<th>Lymph node of the large intestine</th>
<th>Pulmonary lymph node</th>
<th>Lung</th>
<th>Spleen</th>
<th>Testicle</th>
<th>Thymus</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEHV-1A</td>
<td>ND</td>
<td>1.22 × 10^4</td>
<td>ND</td>
<td>2.02 × 10^4</td>
<td>ND</td>
<td>7.53 × 10^3</td>
<td>5.41 × 10^4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>EEHV-4</td>
<td>2.81 × 10^5</td>
<td>1.10 × 10^4</td>
<td>1.60 × 10^4</td>
<td>3.38 × 10^8</td>
<td>5.65 × 10^3</td>
<td>5.01 × 10^4</td>
<td>1.16 × 10^4</td>
<td>8.04 × 10^4</td>
<td>4.07 × 10^4</td>
</tr>
</tbody>
</table>

ND, Not detectable.
this resulted from variable stability of the two viruses and longer-term storage of the samples is yet to be explored.

Discussion
Detection of more than one herpesvirus from clinical specimens is not uncommon. Co-infections of humans with herpesviruses in the subfamily Betaherpesvirinae, the closest to EEEVs, have been reported for human herpesvirus (HHV)-5 (human cytomegalovirus), -6A, -6B and -7 (Tang et al., 1997; Fillatre et al., 2013; Ahmed, 2014). In Asian and African elephants, co-infections with several EEEVs, EEEVs and elephant gammaherpesviruses (EGHV), as well as co-infections with several EGHVs have also been described (Latimer et al., 2011; Ortega et al., 2015). However, there are no reports of simultaneous detectable proboscivirus infections in clinical infections. Stanton et al. (2013) recently suggested the possibility of concurrent and subclinical infections of elephants with different EEHV strains, and the data presented here provide the first evidence of such EEHV co-infection. Dual herpesvirus infections in humans have been suggested to increase the risk of viral syndromes and disease development (Chapenko et al., 2000); therefore, it seems to be of considerable value to screen elephants for multiple herpesviruses both for routine monitoring and disease investigation.

The clinical picture as well as the pathological findings of the EEHV-1A and -4 co-infection in the case reported here were overall similar to those described for fatalities caused by EEHV-1, -3 and -5 (Garner et al., 2009; Wilkie et al., 2014). However, characteristic endothelial herpesvirus inclusions were unusually prevalent, particularly in the larger vessels of the myocardium, and the amount of haemorrhage in the intestinal mucosa and the presence of whole blood in the ingesta has not been a classical finding. Interestingly, very low mitotic activity was observed in the intestinal crypts along with central lymphoid necrosis of the Peyer’s patches, suggesting a radiomimetic effect, which has not been previously reported in EEHV fatalities.

In conclusion, we have presented a fatal case of EEHV co-infection, described its histopathological manifestations and determined the viruses involved. The overall findings confirm EEHV-1A and -4 to be the cause of death in this elephant. To the best of our knowledge, this is the first documented fatality of a juvenile Asian elephant caused by co-infection of two pathogenic genotypes of EEHV, although with substantial differences in organ viral load. This case is also the first fatality in a European zoo from which EEHV-4 could be identified. Detection of EEHV-4 in this fatal case in Europe underlines the importance of inclusion of all known Asian elephant EEHVs in routine blood monitoring to facilitate early therapeutic intervention. Finally, observation of the highest EEHV-1A and -4 loads, coinciding with pathological changes in lymphoid tissues of the intestinal tract, suggest that immune cells of the intestinal tract lymphoid tissues could be a site for viral latency and/or initial replication of the virus.

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References

