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Fakulteten för veterinärmedicin och husdjursvetenskap
Institutionen för biomedicin och veterinär folkhälsovetenskap

Detection of elephant endotheliotropic herpesvirus (EEHV) in Asian (*Elephas maximus*) and African elephants (*Loxodonta africana*)

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Diagnosticering av elefantens endoteliotropa herpesvirus (EEHV) hos asiatiska (*Elephas maximus*) och afrikanska elefanter (*Loxodonta africana*)

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Index

ABSTRACT

Elephant endotheliotropic herpesvirus (EEHV) has been identified as the cause of rapid onset acute hemorrhagic disease. EEHV infects both Asian (*Elephas maximus*) and African elephants (*Loxodonta africana*). The virus has been detected in wild and captive populations. Seven different genotypes of EEHV have been identified so far. EEHV1 is recognized as the most common cause of acute disease, and more rarely EEHV3 and EEHV4. EEHV-associated disease is most common in juvenile Asian elephants. Primary infection can be either subclinical or cause the disease. EEHV can persist in the host in latent phase and reactivate from time to time. Data collection from ex situ elephants worldwide, suggest that subclinical EEHV1 infections are common in captivity. Subclinical infections may occur in situ also. An elephant with subclinical infection can shed the virus particles into a range of the host fluids. The infected fluids may have the potential to transmit a virus to other elephants in contact with the host. To be able to manage this devastating disease it is essential to identify animals with subclinical or latent infections. It is also critical to diagnose animals with acute disease immediately after the first symptoms appear. Reliable, specific, fast and easy to use diagnostic tools are much needed. Currently available diagnostic tools and new possible methods such as quantitative real-time PCR, antibody based detection, acute phase protein expression, viral microRNA detection and ultrasound-guided biopsy sampling are described in this review.

SAMMANFATTNING

Endotheliotropiskt herpesvirus (EEHV) hos elefant har påvisats som orsak till en snabbt uppkommande akut hemorragisk sjukdom. EEHV infekterar både asiatiska (*Elephas maximus*) och afrikanska (*Loxodonta africana*) elefanter. Viruset har påvisats både hos elefanter i det vilda och i fångenskap. Idag har sju olika genotyper av EEHV identifierats. EEHV1 är den vanligaste orsaken till akut sjukdom medan EEHV3 och EEHV4 orsakar detta mer sällan. EEHV-associerad sjukdom är vanligast hos juvenila asiatiska elefanter. Den primära infektionen kan antingen vara subklinisk eller orsaka sjukdom. EEHV kan kvarstå i värden i en latent fas och kan återaktiveras vid olika tillfällen. Tester av elefanter från olika platser visar att subkliniska EEHV1-infektioner är vanliga i fångenskap men subkliniska infektioner har också inträffat i det vilda. En elefant med subklinisk infektion kan utsöndra viruspartiklarna till ett antal olika kroppsvätskor och sprida virus till andra elefanter som kommer i kontakt med värden. För att kunna hantera denna förödande sjukdom är det viktigt att identifiera djur med subkliniska eller latenta infektioner. Det är också viktigt att diagnosticera patienter med akut sjukdom omedelbart efter att de första symptomen uppkommer. Det finns ett stort behov av säkra, specifika, snabba och användarvänliga diagnostiska metoder. För närvarande tillgängliga diagnostiska metoder och nya tänkbara diagnostiska metoder såsom kvantitativ realtids-PCR, antikroppsbaserad detektion, akutfasprotein uttryck, viral mikroRNA detektering och ultraljud guidad biopsi provtagning beskrivs i detta arbete.

INTRODUCTION

Over 90 cases of suspected elephant endotheliotropic herpesvirus (EEHV)-associated hemorrhagic diseases have been reported in elephants worldwide, of which at least 57 cases have been confirmed with DNA PCR diagnostics to involve EEHV infection (Fickel *et al.*, 2001, Garner *et al.*, 2009, Ossent *et al.*, 1990, Richman *et al.*, 1999, 2000 and 2014, Zacharian *et al.*, 2008). EEHV-associated hemorrhagic disease has rapid pathogenesis and a high fatality rate of about 80% (Hardman *et al.*, 2012, Hayward, 2012). Disease is most common in young, captive-born Asian elephants (*Elephas maximus*). Deaths due to EEHV-associated disease cover approximately 65% of the overall mortality rate of captive-born Asian elephants in North America (Hayward, 2012).

Over the last 15 years, several diagnostic tools have been developed to detect active EEHV infections in elephants (Humphreys *et al.*, 2015, Latimer *et al.*, 2011, Stanton *et al.*, 2010, 2012 and 2014, Zacharian *et al.*, 2013). Diagnostic real-time quantitative PCR (qPCR) sets have been developed and have been evaluated to be sensitive and specific to detect various genotypes and subgenotypes of EEHV (Stanton *et al.*, 2012). EEHV have been detected from elephant whole blood, tissue samples, conjunctival swabs, palate, vaginal mucosa, urine, and trunk secretion (Hardman *et al.*, 2012, Shaftenaar *et al.*, 2010, Stanton *et al.*, 2013a and 2014, Zachariah *et al.*, 2013). Attempts to develop diagnostic tools that identify EEHV carriers with latent infection have also been made. Possibilities of using antibody responses, microRNA detection, and lymph node biopsy samples have been explored (Furuse *et al.*, 2014, Gao *et al.*, 1999, Hildebrand *et al.*, 2005, Kania *et al.*, 1997, Kelly *et al.*, 1998, Speckner *et al.*, 1999, Stanton *et al.*, 2012).

The following paper reviews different diagnostic approaches to identify EEHV infected individuals and the epidemiology and transmission of this virus.

MATERIALS AND METHODS

Pub Med and Web Of Sciences databases were used to search articles.

Example of search phrase:
(EEHV OR Elephant endotheliotropic herpesvirus OR Elephant herpesvirus OR Elephant gammaherpesvirus)

LITERATURE REVIEW

Asian and African elephant (*Loxodonta africana*) are both on the International Union of Nature's (IUCN) Red List of endangered species. EEHV associated disease is devastatingly severe and survival of these charismatic animals is under extreme pressure in both wild range countries and in captivity (Hayward, 2012, Zachariah *et al.*, 2013).

Herpesvirus in Asian and African elephants

Herpesviruses

Herpesviruses are double-stranded DNA (dsDNA) viruses classified in Baltimore's class I. Over 100 herpesviruses have been identified to belong in the order of *Herpesviriadae*. A notable characteristic of herpesviruses is the ability to persist in the infected host, often in latent form, and reactivate from time to time. Both reactivated and primary infections can be either asymptomatic or result in disease (Carter and Saunders, 2013). Herpesviruses have among the largest and most variable of all DNA genomes and are overall among the most extensively studied large DNA viruses (McGeoch *et al.*, 2006, Richman *et al.* 2014). Detailed analysis of capsid structures has provided strong evidence of a common ancestor for the group containing herpesviruses naturally hosted by mammals, birds and reptiles (McGeoch *et al.*, 2006).

The viral replication cycle

Herpesvirus particles have large numbers of proteins organized into three structures: capsid, tegument, and envelope. The genome is a linear dsDNA molecule, which both strands are used for coding. The genome is housed in the icosahedral, enveloped capsid. Depending on the cell type, infection can occur by endocytosis or virion envelope fuses with plasma membrane. After virion reaches the cytoplasm some tegument proteins are released. Others teguments stay associated with the nucleocapsid to be transported via microtubules into the nucleus, where the virus replication takes place (Carter and Saunders, 2013).

Herpesvirus genes are expressed in three phases: immediate early (IE), early (E), and late phase (L). IE genes promote transcription of E and L genes and protect the virus from the host immunity. Early viral mRNA is transcribed by host polymerase II, encoding E proteins that are essential for virus DNA replication. It is believed that the first round of circular genome amplification occurs via bidirectional replication and later switches to rolling circle replication mode. L proteins are used to form the capsid and accumulate in the nuclear replication compartments (Carter and Saunders, 2013).

During assembly, DNA is packed first into procapsid and then nucleocapsid is constructed. Nucleocapsid acquires its teguments and host-derived envelope and is then released from the cell. Virus glycoproteins are expressed on the infected cells surface and can fuse infected cells with non-infected cells. This can result in giant cells known as syncytia, and are observed in herpes lesions (Carter and Saunders, 2013).

Viral latency

Latent herpesvirus infection results when virus genome is maintained in the host nucleus without productivity. For most herpesviruses multiple copies of the genome stay in circular formation in the nucleus, but some herpesviruses get their genome integrated into the host chromosome. Most of the virus genome is switched off during the latent period, but a few regions are transcribed into RNA and some viruses synthesize a few proteins. If the host cell is a neuron it does not divide and no proteins are required to maintain the latency in the cell. In the neuron some RNA is synthesized, known as latency-associated transcripts (LATs). LATs give rise to two shorter LATs and microRNAs, which participate in inhibition of apoptosis so the neuron survives. If the host cell does divide, proteins are needed to maintain the copy number of the virus genome in new cells and proteins are synthesized. Latent herpesvirus can reactivate with increased likelihood if the host becomes immunocompromised (Carter and Saunders, 2013).

Elephant endotheliotropic herpesvirus

Viral genomic DNA of EEHV is well studied despite the fact that EEHV has not yet been grown successfully in cell culture. The complete genome sequence of two subgenotypes EEHV1A, EEHV1B and genotype EEHV5 have been completely resolved (Wilkie *et al.*, 2013, Wilkie *et al.*, 2014). Seven distinct EEHV genotypes have been identified based on viral DNA sequences analysis. EEHV2, EEHV3, EEHV4, EEHV5, and EEHV6 differ 15-35% on the nucleotide level in several genes and from each other by 8-35% (Latimer *et al.*, 2011, Richman and Hayward 2011, Stanton *et al.*, 2012). EEHV1A and EEHV1B are complex partially related chimeric subspecies, which differ greatly in two non-adjacent loci but are largely very similar (Ehlers *et al.*, 2001, 2006, Ficker *et al.*, 2001).

Until the recent, EEHVs have been classified in the genus *Proboscivirus*, subfamily of *Betaherpesvirinae*, family *Herpesviridae*, order *Herpesvirales*. Richman *et al.* (2014) suggested that EEHV1A, EEHV1B and EEHV2 are so highly diverged from the other mammalian herpesviruses that they may form a new subfamily of *Deltaherpesvirinae*. Five elephant gammaherpesviruses (EGHVs) from the subfamily of *Gammaherpesvirinae* have been reported; EGHV1-5, and subgenotypes EGHV3A and EGHV3B (Latimer *et al.*, 2011, Wellehan *et al.*, 2008). EGHVs have been detected from vaginal and conjunctival swabs of asymptomatic Asian and African elephants, but none have yet been suspected of contributing to systemic disease (Ehlers *et al.*, 2008, Latimer *et al.*, 2011).

Disease

EEHV-associated disease ranges in spectrum from subclinical infection to rapidly fatal hemorrhagic disease (Richman *et al.*, 1999). It is believed that EEHV, like many viruses in the family of *Herpesviridae*, has the ability to cause mild asymptomatic or systemic primary infection and then persist in a quiescent latent phase. Latent infection occasionally reactivates as localized skin or mucosal epithelial lesions with active lytic infection (Carter and Saunders, 2013, Stanton *et al.*, 2010).

In systemic disease EEHV causes rapid and extensive damage to endothelial cells and often results in per-acute or acute death (Richman *et al.*, 1999). The disease has very rapid onset and is especially serious in young captive Asian elephants (Richman *et al.*, 1999 and 2000, Garner *et al.*, 2009). Studies of the asymptomatic elephants have shown subclinical EEHV1, EEHV3, EEHV4, EEHV5 infections among Asian elephants and EEHV2, EEHV3, EEHV6 and EEHV7 infections in African elephants. These EEHVs can be detected in whole blood, serum, tissue samples, conjunctival swabs, palate, vaginal mucosa swabs, trunk secretion and urine. This suggests that elephants can be persistently infected with these EEHV subtypes (Shaftenaar *et al.*, 2010, Hardman *et al.*, 2012, Zachariah *et al.*, 2013, Stanton *et al.*, 2013a and 2014).

It has been suggested that EEHV1, like other known betaherpesviruses, may infect a subset of leukocytes and becomes detectable in whole blood. Subsequently, active viral replication has been shown to persist for at least several weeks in some cell type involved in producing nasal secretions before presumably establishing long-term quiescent latency (Stanton *et al.*, 2013b). It is not known in which cells and where the EEHV rests in latent form. The mouth is a presumed site of infection and it has been hypothesized that lymph node draining the mouth could be a possible location. However, the attempt to detect latent EEHVs has not yet been successful (Hildebrand *et al.*, 2005).

Fatality

EEHVs cause lethal hemorrhagic disease in both Asian and African elephants (Ehler *et al.*, 2001, Fickel *et al.*, 2001, Garner *et al.*, 2009, Ossent *et al.*, 1990, Richman *et al.*, 1999), but appear to be more pathogenic in Asian elephants (Fickel *et al.*, 2001, Hardman *et al.*, 2012). EEHV-associated disease has a mortality rate of about 80% in the nearly 90 known infections in Asian elephants that have been reported over the last 20 years. The disease is especially severe in young animals and significant numbers of otherwise healthy captive-bred calves have succumbed to EEHVs (Hayward, 2012, Hardman *et al.*, 2012). There are only three known cases of fatal EEHV infection in African elephants (Hayward, 2012). EEHV1A and EEHV1B subgenotypes have been responsible for the majority of the EEHV-associated deaths in Asian elephants (Garner *et al.*, 2009, Latimer et al, 2011, Richman *et al.*, 1999). EEHV3, EEHV4 and the recently reported EEHV5, are known to cause sporadic fatalities in young Asian elephants, and EEHV2 has been attributed to similar disease in African elephants (Richman *et al.*, 1999, Stanton *et al.*, 2014, Wilkie *et al.*, 2014). EEHV has been attributed to be the cause of death in wild elephants, but the number of incidents in the wild is still unknown (Latimer *et al.*, 2011, Richman *et al.*, 1999, Stanton *et al.*, 2014).

Origin

Subclinical presentation of EEHV, with virions detected in pulmonary lymphoid nodules and cutaneous papillomas, has been documented in high numbers of wild African elephants as far back as in the 1970's (McCully *et al.*, 1971). A relatively small number of EEHV-associated diseases have been reported in African elephants (Hayward, 2012). Therefore, it was originally suggested that EEHV1 is indigenous to and nonlethal in African elephants, and that disease in Asian elephants resulted from cross-species transmission (Richman *et al.*, 1999).

However, a high level of EEHV1 subtype diversity have been recently found among Indian strains that matched with the strain diversity found in Europe and North America. This suggests that it is more likely that the Asian elephant is an ancient endogenous host of EEHV1, and the virus is widespread and transmitted between Asian elephant populations worldwide (Zacharian *et al.*, 2013). Genotypes EEHV2 and EEHV6 have not been detected from the Asian elephants. The question is still open if some of the EEHV genotypes are native to African elephants and some to Asian elephant (Stanton *et al.*, 2014).

Transmission

In a recent study by Stanton (2013b), EEHV1 kinetics in viral loads have proven EEHV genotypes that can produce DNAemia (viral DNA within the bloodstream) are subsequently shed in trunk secretions for at least several weeks before presumably establishing long-term quiescent latency. The actual pathogenesis of the infection and the nature of EEHV1 persistence, latency and reactivation remain to be determined. The actual transmission action is suspected to only happen through contact of the trunk with genitals and then with the mouth most likely during greeting ceremonies (Hildebrandt *et al.*, 2005, Stanton *et al.* 2013b).

Multiple infections

There is evidence that one individual elephant can acquire multiple EEHV infections, suggesting that prior infection with another EEHV subtype does not confer sterilizing immunity against another EEHV subtype (Stanton *et al.* 2013b, Richman 2014). In several cases, elephants have been showing evidence of both EEHV1A and EEHV1B subgenotypes, but have not been observed to be simultaneously infected or shedding the two. It seemed that the first subtype was cleared to undetectable levels in blood and nasal secretions before detection of a new infection episode with the second subtype (Stanton *et al.* 2013b).

Little is known about the pathogenicity of the EEHV and there is a possibility that multiple EEHV infections and EGHV infections can be an underlying cause resulting in the disease (Latimer *et al.*, 2011). Likely multiple host factors are playing a role including age of exposure, maternal antibodies during gestation or nursing, latent infections and preexisting presence of immunocompromising disease states (Stanton *et al.*, 2010). Whether preexisting infection with another EEHV confers enough immunity to prevent fatal disease is not yet known (Stanton *et al.* 2013b).

Treatment

EEHV-associated hemorrhagic disease is treated with herpesvirus specific antiviral medication famciclovir or ganciclovir (Richman *et al.*, 2000, Brock *et al.*, 2002). Famciclovir is a prodrug that is rapidly deacetylated and oxidated to active form penciclovir. Penciclovir and ganciclovir are acyclic nucleoside analogues, which target viral DNA polymerase (Brock *et al.*, 2002). Famciclovir can be administered rectally or orally being effectively absorbed and have similar extents of exposure. A dose of 8 to 15 mg of famciclovir/kg every 8 hours for the treatment of Asian elephants is recommended (Brock *et al.*, 2002). To be able to interact with viral DNA synthesis penciclovir and ganciclovir need to be phosphorylated

intracellularly to the triphosphate form. The phosphorylation step is ensured by herpesvirus encoded kinase, and is therefore confined to virus-infected cells. This explains the specific antiherpetic ability of the compounds. Nucleoside analogue in its triphosphate form interact with viral DNA polymerase as a chain terminator and inhibits viral DNA synthesis (Brock *et al.*, 2002). There are only 10 known Asian elephants that survived the symptomatic diseased, which were all treated aggressively with antiviral drugs, although such treatments were not effective in many other cases (Hayward, 2012).

Clinical symptoms

Systemic disease

The systemic disease attributed to EEHV has a sudden onset of an acute hemorrhagic disease (Richman *et al.*, 1999; Stanton *et al.*, 2014). The clinical course may last from one to seven days and initial clinical signs include lethargy, anorexia, mild colic, and edema of the head and thoracic limbs. In the later course of the disease, swelling and cyanosis of the tongue is noted (Richman *et al.*, 2000). EEHV viral DNA can be detected in the peripheral blood with a typical viral load of 10^4 viral genomic copies (VGCs) per ml of whole blood when the clinical signs start (Stanton *et al.*, 2010). Haematological changes include decreased white blood cell and platelet count (Richman *et al.*, 1999). Symptoms such as limb stiffness or lameness and non-endothelial, cutaneous lesions are also possible (Ossent *et al.*, 1990, Schaftenaar *et al.*, 2009). Untreated systemic disease ultimately leads to a myocardial failure and death (Richman *et al.* 1999, Richman *et al.*, 2000).

Nonfatal clinical presentation

EEHVs have been isolated from skin papillomas, vestibular lymphoid patches and pulmonary nodules of otherwise healthy Asian and African elephants that were not exhibiting systemic disease (McCully *et al.*, 1971, Richman *et al.*, 1999, Schaftenaar *et al.*, 2009). Transmission from these active lesions to susceptible elephant could easily occur, and possibly cause either asymptomatic infection or systemic disease for new hosts (Schaftenaar *et al.*, 2009).

Pathological findings

Pathological findings in fatal cases associated with EEHV genotypes that are recognized today are typically similar, consisting of extensive edema, effusions, and hemorrhages. However, in EEHV1 fatal cases the primary appearance site has been the cardiovascular system and recent cases of EEHV3, EEHV4 and EEHV5 have suggested less selective organ tropism (Garner *et al.*, 2009).

Tropism

EEHV3 infection has been related to renal medullary hemorrhage, retinal damage, and tropism for larger veins and arteries (Garner *et al.*, 2009). EEHV4 has demonstrated hemorrhages in most organs, including gastrointestinal, respiratory, and cardiovascular systems (Sripiboon *et al.*, 2013). Widespread hemorrhages and edema have been found in the EEHV5 case and it has been suggested that EEHV3, EEHV4 and EEHV5 might reflect higher

virulence of the strain involved (Garner *et al.*, 2009, Sripiboon *et al.*, 2013, Wilkie *et al.*, 2014).

Gross findings

Typical gross findings are pericardial effusion with extensive petechial to ecchymotic hemorrhages involving the epi- and endocardial heart surface and throughout the myocardium. All of the visceral and parietal peritoneal serous membranes are with scattered petechiae. Hepatomegaly, and variable cyanosis of the tongue, oral, laryngeal and large intestinal ulcers can be found (Richman *et al.*, 2000).

Histological findings

Histological findings include extensive micro-hemorrhages and edema throughout the myocardium and mild subacute myocarditis. Hemorrhagic lesions with inflammation have been found in the target organs and were accompanied by amphophilic to basophilic intranuclear inclusion bodies in capillary endothelial cells (Richman *et al.*, 2000). EEHV inclusion bodies have been detected in the heart, lungs, and liver and in the case of EEHV5 also in the adrenal gland and oral mucosa (Garner *et al.*, 2009, Wilkie *et al.*, 2014). High levels of viral DNA are found in white blood cells, serum, and necropsy tissues (Latimer *et al.*, 2011).

Diagnostics

Quantitative real-time PCR

There have been several reports on polymerase chain reaction (PCR) based methods developed to detect viral DNA in varied fluid and histological samples (Fickel *et al.*, 2001 and 2003, Garner *et al.*, 2009, Schaftenaar *et al.*, 2009, Hardman *et al.*, 2012, Latimer *et al.*, 2011, Sariya *et al.*, 2012, Stanton *et al.*, 2010 and 2014). Viral DNA can be detected before the onset of clinical illness and peak in whole-blood viral loads occur 3-8 days after the onset of clinical signs (Stanton *et al.*, 2013b).

Sample collection and preparation

Trunk-wash samples can be collected by means of the standard recommended technique for collection of samples for *Mycobacterium tuberculosis* testing by the USDA. Sterile saline solution is poured to the nares of the elephant, and the proboscis is elevated for about half a minute, after which the elephant blows the effluent to a container (Stanton *et al.*, 2010). The sample is then centrifuged into a cell pellet, which is processed with a commercial kit to extract the DNA (Stanton *et al.*, 2010, Hardman *et al.*, 2012).

Swab samples collected from the trunk can be stored in viral transport medium (VTM) that can be supplemented with gelatin and antibiotics (Sariya *et al.*, 2012). Swab samples from conjunctiva, palate and vulva can be collected by using swabs moistened with phosphate buffer solution or sterile saline and later soaked in phosphate buffer solution (Hardman *et al.*, 2012). In the laboratory, swab samples are centrifuged and stirred. The swab is removed and centrifugation is repeated. The cell pellet is then processed with a commercial kit (Sariya *et*

al., 2012). When processing tissue- and whole blood samples DNA is extracted by using a commercial kit to receive first a cell pellet and then extracted DNA (Stanton *et al.*, 2010).

DNA amplification

The extracted DNA from the pellets can then be quantified with a spectrophotometer. Samples are used either directly or amplified by using a commercial kit before being subjected to the real-time PCR (Hardman *et al.*, 2012).

qPCR assays

Primers and probes that are designed for published sequences of EEHVs are available in the GenBank with accession numbers (NAPs) (Hardman *et al.*, 2012, Stanton *et al.*, 2010). Sensitive qPCR assays have been developed for EEHV1A and 1B, EEHV2, 3, 4, 5, and 6 (Stanton *et al.*, 2012). Asian elephant tumor necrosis factor alpha (TNF) assay can be included to the PCR run to detect Asian elephant genomic DNA. It can be used as an internal PCR amplification control to determine if taken samples do contain amplifiable elephant genomic DNA (Stanton *et al.*, 2010 and 2012).

Reliability

It has been shown that available EEHV sequence-specific hydrolysis probes are highly specific, and the chance of amplifying non-EEHV nucleic acids is likely small (Stanton *et al.*, 2014). QPCR assays designed to recognize specific sequence in either the DNA polymerase gene or the terminase gene for EEHV2-6 types have been proven to have high specificity, sensitivity and reproductive ability. The only assay that has exhibited cross-reactivity between different EEHV species has been EEHV3/4 assay. Therefore a single qPCR assay is used to detect EEHV3/4. Reasons for cross-reactivity are the high DNA sequence similarity between EEHV3 and EEHV4 amplicon sequences and the primer design used. EEHV3 and EEHV4 have 100% identical terminase gene (Stanton *et al.*, 2012).

Developed EEHV qPCR assay has been shown to be reliable detecting virus DNA in a wide variety of selected samples including tissue taken from necropsy and blood from symptomatic animals as well as trunk washes or blood samples from the asymptomatic animals (Stanton *et al.*, 2010 and 2014). In Hardman *et al.* (2012) swab samples collected from conjunctiva, palate, and vulva were compared with trunk wash samples. EEHV1 was detected at the highest rate from the conjunctival swabs. The result does not correlate with Stanton *et al.* (2014), which did not detect any EEHV's from conjunctival swab samples. It is possible that this may be a result from different DNA purification methods, QPCR assays, and qPCR reagents that were used (Stanton *et al.*, 2014).

Antibody-based detection

Ehlers *et al.* (2001) have identified a glycoprotein B (gB) gene in EEHV1. GB is a major herpesviral envelope protein known to induce a neutralizing antibody response in herpesvirus infections (Gao *et al.*, 1999, Speckner *et al.*, 1999). In a recent study, Humphreys *et al.* (2015) were not able to express gB homolog but instead achieved robust expression of another surface glycoprotein L (gL). First generation enzyme-linked immunosorbent (ELISA) assay

was set up to detect anti-EEHV1 gL antibodies from elephants that were known to be previously infected with EEHV1, and relatively constant positive titers were achieved (Humphreys *et al.*, 2015).

Three elephant immunoglobulin isotypes have been detected so far; immunoglobulin G (IgG) (Kania *et al.*, 1997, Kelly *et al.*, 1998) in both Asian and African elephants and immunoglobulin A (IgA) and M (IgM) in Asian elephants (Humphreys *et al.*, 2015). Specific antisera reactive against IgA and IgM isotypes, and monoclonal antibody against Asian elephant IgG have been generated. All three immunoglobulin isotypes can be detected in Asian elephant serum and milk. These reagents might be useful in the future for developing sensitive and specific assays to detect elephant antibody responses for EEHV and other pathogens or possibly even vaccines (Humphreys *et al.*, 2015).

Acute phase protein expression

The acute phase response acts as a major part in inflammatory response and is mediated by acute phase proteins (APP). Serum amyloid A (SAA) has been identified as a major APP in elephants (Stanton *et al.*, 2013a). Infection, inflammation, stress, neoplasia and trauma can give rise to APPs, which are synthesized within 24 hours of the stimuli and can increase in magnitude rapidly (Kjelgaard-Hansen *et al.*, 2003, Crey *et al.*, 2009 and 2011). It has been demonstrated that SAA levels from elephants with EEHV1 viremia are significantly increased, but APPs are nonspecific biomarkers with a short half-life (Kjelgaard-Hansen *et al.*, 2003, Stanton *et al.*, 2013a). At the moment SAA levels have potential in health examinations of elephants or for establishing a prognosis upon infection of EEHV (Stanton el al., 2012).

Viral microRNA detection

MicroRNAs (miRNA) are regulatory RNAs which post-transcriptionally down-regulate the expression of target mRNAs (Bartel, 2009). Many herpesviruses, including many in hte Betaherpesvirinae family are known to encode viral miRNAs, which are often expressed at very high levels and released to the serum (Cullen, 2009). It has been demonstrated that EEHV1 expresses at least three novel viral miRNAs of which miR-E3-5P was shown to be functional and expressed at high levels in the infected cells. MiR-E3-5P has been detected from a whole blood sample of an EEHV1 positive elephant by using miR-E3-5P specific TaqMan probe. It is possible that miR-E3-5p is also released into the circulation of animals that are asymptomatically infected with EEHV1 and can be used as a biomarker if the level is high enough to be detected (Furuse *et al.*, 2014).

Ultrasound-guided biopsy

Biopsy samples from lymphoid tissue appears to be one option for detecting latently EEHV infected lymphocytes. Hildebrand *et al.* (2005) have identified a lateral retropharyngeal lymph node in elephants, that collects lymphatic drainage from the mouth, specifically from the salivary gland, gingiva and tongue, which are presumed to be initial site of EEHV infection (Hildebrand *et al.*, 2005).

Ultrasound-guided, fine-needle biopsy sampling has been successfully completed for elephants. Active, inactive and chronically active lymph tissues were identified, but no EEHV DNA or occlusion bodies were found. Reactivity status can provide information about overall health status of the elephant but is unspecific about the cause of activation. It is possible that a lymph node biopsy procedure may prove beneficial in the future and the retropharyngeal lymph node can be a site of latent EEHVs (Hildebrand *et al.*, 2005)

DISCUSSION

The overall prevalence of EEHV infection in elephants throughout the world is still not known but is expected to be high (Stanton *et al.*, 2014). What is notable is that routine investigations, in captive populations in particular, focus on the detection of EEHV1 and tend to neglect other genotypes (Wilkie *et al.*, 2014). If only EEHV1 is tested, it is possible that the lower fatality reports associated with other genotypes do not necessarily indicate lower prevalence of these viruses, but are due to the appearance of virulent variants. There are apparent occurrences of a recombination between EEHV1 subgenotypes. A recombination could render divisions of other EEHV genotypes to subgenotypes. This would be problematic with PCR assays that are based on different gene forms from known EEHVs (Wilkie *et al.*, 2014).

A difficult management issue for EEHV-associated disease is its rapid progression and short time frame between appearance of clinical signs and death, which leaves little time for diagnostics (Ossent *et al.*, 1990). Detection of latent carriers would help caretakers prevent infection of naïve animals and identify animals with a need for regular monitoring. Regular monitoring would lead to earlier disease diagnosis in cases of EEHV reactivation and treatment could begin on time. QPCR diagnostic assays are the most used method in practice and can detect EEHV viral DNA from several different kinds of fluid samples. An issue with EEHV viral DNA detection from the body fluids is that EEHV has to be in an active stage (Stanton *et al.*, 2012).

Recently generated first generation gL ELISA assay was able to detect anti-EEHV1 antibodies from a serum sample of Asian elephants (Humphreays *et al.*, 2015). This gL ELISA assay could be a potential diagnostic tool for identifying the asymptomatic and latent carrier elephants that have been exposed to EEHV1. It appears as a most promising diagnostic tool for the future to perform infection monitoring and epidemiological studies. Antibody assay would also be valuable for evaluating potential treatments or vaccines against EEHV infection.

Another diagnostics method with potential to detect latent carriers in the future is miRNA expression detection. Furuse *et al.* (2014) were able to identify one functional EEHV1 miRNA (miR-E3-5p) and two other possible EEHV1 miRNA, from whole blood and several tissue samples of EEHV1 infected elephants. MiRNAs expression in the elephants have not yet been studied extensively, but it is possible that viral miRNAs could target the same mRNAs that are targeted by cellular miRNA and be expressed during the viral latency. It is

unlikely that they represent targets for intervention in EEHV-associated disease, but MiRNAs might have the potential to serve as biomarkers in diagnostics (Furuse *et al.*, 2014).

Lymph node biopsy procedures may prove beneficial in the future for screening diseases in elephants. In vivo assessment and biopsy of the lymph nodes are standard procedures in domestic animals. Many disease processes are diagnosed and evaluated by the examination of lymph nodes (Hildebrant *et al.*, 2005). Previously, only few lymph nodes had been identified from the elephant. The retropharyngeal lymph node that Hildebrand *et al.* (2005) identified from the elephant is now known to be accessible in vivo. It was stated that guidance of the ultrasound in the procedure is essential, since the lymph node cannot be palpated or visualized externally. There is also a risk of puncturing the associated blood vessel causing uncontrollable bleeding, if the area is not visual (Hildebrand *et al.*, 2005). Biopsy collection is a difficult procedure, especially in the wild, and fast reliable detection methods are much needed for control of the EEHVs.

Collection of conjunctival swabs have been found to be less difficult than collection of trunk washes, suggesting that optimization of EEHV screening using conjunctival or other mucosal swabs would be useful especially for screening of non-trained animals (Stanton *et al.*, 2014). Additional diagnostic assays designed for swab samples and serological anti-EEHV antibody assays for all EEHV genotypes would make routine monitoring of the animals remarkably easier. Recent study results have been encouraging and these tools may be available in the near future (Humphreys *et al.*, 2015).

There is a lack of knowledge about the conditions under which EEHVs cause fatal hemorrhagic disease and the conditions in which latent infections are reactivated. Herpeviruses in general tend to get reactivated from the latent phase more likely if the host becomes immunocompromised (Carter and Sounder, 2013). In pathological examinations from EEHV deceased elephants no evidence of other underlying diseases or infections have been found and cross reactivity with other herpesviruses has not been detected (Ossent *et al.*, 1999, Richman *et al.*, 2000). Hypovitaminosis E have been considered as a differential diagnosis of several elephants due to ecchymotic hemorrhages that were documented at necropsy of several elephants, but low vitamin E levels have not been found (Richman *et al.*, 2000). Co-infections with gammaherpesviruses and several EEHV subtypes have been reported, but the underlying cause remains speculative (Wilkie *et al.*, 2014).

A long-term solution for captive elephants will most likely be the development of adapted annulated live or killed EEHV vaccines. Vaccine development is challenged by the inability to grow any EEHVs in cell culture and also a very large number of different EEHV genotypes.

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