Virus Research 220 (2016) 91–96

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Distribution and load of elephant endotheliotropic herpesviruses in tissues from associated fatalities of Asian elephants

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ARTICLE INFO

Article history:
Received 13 January 2016
Accepted 14 April 2016
Available online 19 April 2016

Keywords:
EEHV-1
EEHV-5
EEHV load
EEHV distribution
Herpesvirus

ABSTRACT

Elephant Endotheliotropic Herpesviruses (EEHVs) are the cause of a highly fatal haemorrhagic disease in elephants primarily affecting young Asian elephants (*Elephas maximus*) in both captivity and in the wild. The viruses have emerged as a significant threat to Asian elephant conservation, critically affecting overall sustainability of their population. So far insight into the pathogenesis of EEHV infections has been restricted to examination of EEHV-infected tissues. However, little is known about distribution and burden of the viruses within the organs of fatal cases, crucial elements in the understanding of the virus pathogenesis. This study was therefore undertaken to assess the extent of organ and cell involvement in fatal cases of EEHV-1A, 1B and 5 using a quantitative real-time PCR. EEHV-1 and 5 DNA were detectable in all the tissues examined, albeit with substantial differences in the viral DNA load. The highest EEHV-1A DNA load was observed in the liver, followed by the heart, thymus and tongue. EEHV-1B and 5 showed the highest DNA load in the heart, followed by tongue and liver. This study provides new insights into EEHV pathogenicity and has implications in choice of sample type for disease investigation and virus isolation.

1. Introduction

Elephant Endotheliotropic Herpesvirus genotype 1 (EEHV-1) is one of the major causes of fatality in juvenile Asian elephants (*Elephas maximus*) and considered as a significant threat to the future of Asian elephant populations. The virus is a large 180 kbp DNA virus which belongs to the genus *Proboscivirus* in the subfamily *Betaherpesvirinae* (Davison et al., 2009; Richman and Hayward, 2012; Wilkie et al., 2013), although suggestions that the virus may cluster into a new subfamily are also made (Richman et al., 2014; Zong et al., 2014). To date, three other genotypes of EEHV have also been detected in the Asian elephants: EEHV-3, EEHV-4 and EEHV-5, all with the potential to cause fatal disease in their host (Garner et al., 2009; Denk et al., 2012; Sripiboon et al., 2013; Seilern-Moy et al., 2015). Phylogenetically, the viruses are most closely related to, but still highly diverged from the human cytomegalovirus (HCMV) and the three human Roseolo viruses (HHV-6A, HHV-6B, and HHV-7), all members of the subfamily *Betaherpesvirinae* (Wilkie et al., 2014).

The viruses' pathology is often associated with generalised haemorrhages and death (Richman et al., 2000). Cardiovascular pathology is the dominant feature displaying extensive haemorrhages on the *epi*- and endocardial surfaces and myocardium and pericardial effusions (Richman et al., 2000; Wilkie et al., 2014). Other pathological attributes are diffuse bleedings within all viscera, cyanosis of the tongue, hepatomegaly, oral, laryngeal and large intestinal ulcers, and haemorrhages in other internal organs. The extent of gross pathological findings can, however, vary drastically depending on the disease duration.

Information on organ and cell tropism and sites of replication and latency are fundamental in understanding pathogenesis of herpesviruses on the whole. Little is known about distribution of the EEHVs within the organs of the fatally affected elephants. Identification of target organs and cells can provide guidance on choice of sample type for virus isolation and disease investigation and shed lights into pathogenesis of the virus. Further, until recently the viral sequences were not resolved and tools to measure viral loads, for example, via qPCR were not available. Here, we have examined distribution and load of EEHV-1 and 5 DNA in several organ materials of associated fatal cases using real-time quantitative PCRs.

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http://dx.doi.org/10.1016/j.virusres.2016.04.012
0168-1702/

Table 1
List of elephants and their respective samples used in this study.

Elephant name	Date of birth	Date of death	Gender	Viral strain	Tissues tested
Raman	12.11.2006	23.07.2009	Male	EEHV-1A	Aorta, blood, heart, kidney, liver, lung, lymph node, parotid, salivary gland, spleen, tongue
Nayan	18.07.2010	29.07.2013	Male	EEHV-1A	Artery, blood, heart, intestine, kidney, liver, lung, mesenterial, mandibular and retropharyngeal lymph nodes, payer's plaque, spleen, thymus, tongue, trunk mucosa
Jamila	22.01.2011	03.07.2013	Female	EEHV-1A	Aorta, blood, bone marrow, heart, intestine, kidney, liver, lung, mesenterial and mediastenal lymph nodes, spleen, thymus, tongue, trunk mucosa
Emelia	16.03.2004	17.12.2006	Female	EEHV-1B	Heart, kidney, liver, lung, retropharyngeal lymph node, spleen, tongue
Vijay	06.08.2009	13.04.2011	Male	EEHV-5	Heart, kidney, liver, lung, lymph node, salivary gland, spleen, thyroid gland, tongue

2. Materials and methods

2.1. Samples and sample processing

The organ samples used in this study were from EEHV-1 and 5 fatal cases of Asian elephants (Table 1). To process the samples for analysis, approximately 3–5 mm^3 of tissue (25 mg) were added to a gentleMACS M tube (Miltenyi Biotec Ltd. UK) containing 600 μl of buffer RLT from EZ1® RNA tissue mini kit (Qiagen, Germany). The tissue was homogenised using the gentleMACS Dissociater (Miltenyi Biotec) and RNA_01 program. The M tube was then centrifuged at 2000*g* for 2 min and 350 μl of lysate were transferred to a 2 ml screw capped tube (supplied in the kit) and 5 μl Internal Control [(High concentration) Qiagen, UK], were added. Total nucleic acid was extracted from the lysate using the EZ1® XL robot (Qiagen) and the EZ1® RNA tissue mini kit for all the tissues. For blood samples, nucleic acid was extracted from 140 μl whole EDTA blood, plus 6 μl of the Internal Control (Qiagen), using the QIAamp® Viral RNA Mini Kit (Qiagen) as described by the manufacturer. The extracted nucleic acid was quantified on a Nanodrop 2000 Spectrophotometer (Agilent Technologies, UK).

2.2. Creating PCR standards

Three conventional PCRs were used to generate DNA standards for EEHV-1, 5 and elephant TNFα in order to quantify viral genome copies in various post mortal organ tissues. The primers used in this study are listed in Table 2. Each PCR was performed in a reaction mix consisting of 10 μl Fast Cycling PCR Master mix (Qiagen), 2 μl DNA template (known EEHV-1 and 5 positive nucleic acid), 0.1 μl of each forward and reverse primers (100 pmol/μl) and RNase-free water to a volume of 20 μl. The thermocycling profile for each PCR was 95 °C for 5 min followed by 35 cycles of 95 °C for 15 s, 55 °C for 30 s and 68 °C for 1 min. The PCR amplicons were then run on an agarose gel (1.5%) and the DNA bands of the correct size were cut out and cleaned using the MinElute Gel Extraction Kit (Qiagen). The purified amplicons were quantified on the Nanodrop 2000 Spectrophotometer (Agilent Technologies), DNA copy numbers were calculated via the fragment size using an online tool http://cels.uri.edu/gsc/cndna.html as per formula: number of copies = [(amount in ng * 6.022 × 10^{23})/(DNA length in bp * 1 × 10^9 * 650)] and DNA concentration was adjusted to 10^6 copies in 2 μl volume and a dilution series ranging from 10^6 to 10^1 copies per 2 μl was created and run in triplicate in the real-time quantitative PCR.

2.3. Real-time quantitative PCR (qPCR)

Various organ samples of EEHV-1A, 1B, and EEHV-5 fatalities (Table 1) were quantified for their viral load using published qPCR protocols for EEHV-1A and B (Hardman et al., 2012). The EEHV-5 real time qPCR primers and probe are listed in Table 3. The nucleic acid from each organ was measured in duplicate for EEHV-1 and 5 and the mean values were calculated using the MxPro (Agilent Technologies) or CFX Manager Software (Bio-Rad, UK). The qPCR was performed using the QuantiFast Pathogen PCR +IC kit using 5 μl 5 × QuantiFast Pathogen Master Mix, 2.5 μl 10 × Internal Control Assay, 0.1 μl of each forward and reverse primers (100 pM/μl), 0.05 μl probe (100 pM/μl), 2 μl template DNA and water to a volume of 25 μl. The qPCR was performed on a Mx3000P (Agilent Technologies) or a CFX 96 Touch™ thermal cycler (Bio-Rad) at 95 °C for 5 min followed by 42 cycles of 95 °C for 15 s, 60 °C for 30 s and signal capture at the end of each cycle.

2.4. Normalisation of viral genome copy numbers

The EEHV genome copy numbers measured in the real-time qPCRs for EEHV and genomic TNFα were first normalised against the spiked Internal Control to compensate for differences in nucleic acid extraction efficiency for each tissue. The EEHV-1 and 5 genome copies for each tissue were then normalised against those of genomic TNFα performed in parallel and 1 μg of extracted DNA.

3. Results

3.1. Performance of the real-time qPCR assay

In order to assess the real-time qPCRs performance and quantify EEHV-1 and 5 genomes in various tissues, several calibration curves were produced with known quantity of DNA standards over a range of 10^6–10^1 copies per PCR mix (Fig. 1). At least 2 independent calibration curves were created for each of the EEHV-1, 5 and TNFα to quantify their genome copy numbers in the tissues. The amplification efficiency was on average 99% (R^2 = 0.999), 98% (R^2 = 0.992) and 95% (R^2 = 0.986) for EEHV-1, 5 and TNFα respectively. The average Ct value for the positive EEHV-1 control included in each experiment was 23 ± 0.45.

3.2. EEHV-1A DNA distribution and load in various tissues

Genomic EEHV-1A DNA was detectable in all the tissues tested (Table 3), albeit with substantial differences in virus DNA load. In a descending order, liver, heart, thymus, tongue, arteries, and blood samples contained the highest viral loads, whereas kidney, spleen, lung, salivary gland, lymph nodes, bone marrow, intestines, payer's plague, and trunk mucosa had the lowest (Fig. 2).

Normalising the EEHV genome copies to those of TNFα, the three elephants which died of EEHV-1A infection (Raman, Nayan and Jamilia) showed the highest viral load in the liver, followed by heart and thymus (Table 3). The ratio of normalised EEHV-1A genome copies for the liver (normalised to TNFα) were 1.7 (Raman), 8.6