Research article

Detection and evaluation of novel herpesviruses in routine and pathological samples from Asian and African elephants: Identification of two new probosciviruses (EEHV5 and EEHV6) and two new gammaherpesviruses (EGHV3B and EGHV5)

Erin Latimer a, Jian-Chao Zong b,1, Sarah Y. Heaggans b,2, Laura K. Richman a, Gary S. Hayward b,*

a Elephant Herpesvirus Laboratory, Smithsonian National Zoological Park, 3001 Connecticut Ave., Washington, DC 20008, USA
b Viral Oncology Program, Johns Hopkins School of Medicine, Baltimore, MD 21231, USA

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ABSTRACT

Systemic infections with elephant endotheliotropic herpesviruses (EEHV) cause a rapid onset acute hemorrhagic disease with an 85% mortality rate. More than 60 cases have been confirmed worldwide occurring predominantly in juvenile Asian elephants. Originally, three virus types EEHV1A, EEHV1B and EEHV2 were identified, all members of the Proboscivirus genus within the Betaherpesvirinae. However, four elephant gammaherpesviruses (EGHV) have also been found by DNA PCR approaches in eye and genital secretions of asymptomatic animals, and two more versions of the probosciviruses, EEHV3 and EEHV4, were recently detected in acute hemorrhagic disease cases. To ask whether even more species of elephant herpesviruses may exist, we have developed several new diagnostic DNA PCR assays using multiple round primers in the DNA POL region. These have been used routinely for nearly three years to screen samples submitted to the Elephant Herpesvirus Laboratory for diagnosis of possible cases of EEHV disease in blood and necropsy tissue, as well as in biopsies of other suspicious lesions or growths. Several more cases of EEHV1-associated hemorrhagic disease were confirmed, but in addition, we describe here eleven examples of other known and novel herpesviruses detected and evaluated with these reagents. They include the prototypes of four new elephant herpesviruses, two more within the proboscivirus group EEHV5 and EEHV6, plus two more gammaherpesviruses EGHV3B and EGHV5. We also report initial semi-quantitative PCR assays demonstrating very high viral loads in the blood of the EEHV3 and EEHV4-associated hemorrhagic disease cases.

1. Introduction

The first herpesviruses described in association with lethal systemic hemorrhagic disease in elephants were referred to as endotheliotropic herpesviruses (EEHV), because of their morphologically visible effects on infected endothelial cells in necropsy tissue. Phylogenetically, they represent a distinctive third major branch of the Betaherpesvirinae (in addition to the Cytomegalovirus/Murmegalovirus and Roseolovirus genera), and have recently...
been assigned to a new genus named Proboscivirus (Davison et al., 2009). EEHV is a significant cause of mortality in zoo, circus and sanctuary elephants, affecting predominantly, but not exclusively, juvenile captive-born Asian elephants (Elephas maximus). Overall, more than 60 confirmed cases of EEHV disease have now been identified, including 33 cases in North America (Richman et al., 1999, 2000a; Garner et al., 2009) and 20 in Europe (Ossent et al., 1990; Ehlers et al., 2001; Fickel et al., 2001, 2003; Ehlers et al., 2006), as well as several in Asia (Reid et al., 2006) (Zachariah, A, pers. comm.). Over 85% of these cases have proved fatal. In North America 60% of all deaths of young captive live-born Asian elephants between 4 months and 15 years of age have been attributed to EEHV. The disease has a very rapid clinical course of just one to five days, involving lethargy, edema and tongue cyanosis, as well as microvascular damage, hemorrhaging and focal necrotic lesions in all major internal organs upon pathological examination (Richman et al., 1999, 2000a; Garner et al., 2009). EEHV-like herpesviruses are not known to exist outside of Elephantid hosts and none have yet been isolated by growth in cell culture.

Two related viruses, EEHV1 and EEHV2, were described originally, based on the DNA sequence of two small PCR products U60/Terminase (=TER) and U38/DNA polymerase (POL) after amplification from whole blood and/or necropsy tissue of captive elephants with this disease (Richman et al., 1999). Until recently, EEHV1 has been implicated in all of the cases in Asian elephants, but two known deaths attributed to a similar disease in African elephants (Loxodonta africana) involved EEHV2 instead. Over the past ten years, DNA PCR testing in whole blood samples of five moribund animals or in post-mortem necropsy tissue has been used routinely in our laboratories as a rapid diagnostic assay to successfully detect acute systemic EEHV1 infection (viremia) in 16 more cases of elephant hemorrhagic disease. However, the same PCR test has been uniformly negative in almost all blood samples from healthy animals as well as in numerous unrelated necropsy samples. Based on the results of this PCR test, at least seven systemically infected juvenile Asian elephants that were treated in a timely fashion with the antiviral drugs famciclovir or ganciclovir after early detection of both known and several previously unknown elephant herpesviruses, we realized the necessity and value of developing several new sets of diagnostic PCR primers designed to improve the sensitivity and specificity for detection of a variety of distinct subsets of elephant herpesviruses. Here we describe a number of additional findings relating to the genetic diversity of known elephant herpesviruses, we have recently described the pathologic changes associated with two new cases of fatal hemorrhagic disease in Asian elephant calves that were not attributed to either EEHV1 or EEHV2 (Garner et al., 2009). Instead, we were able to identify two new elephant endotheleotropic herpesvirus genomes in blood and necropsy tissue that we referred to there as EEHV3A and EEHV3B. These are members of a novel G plus C-rich branch (67% GC-content) of the probosciviruses that is highly diverged from the A plus T-rich branch (42% GC-content) encompassing EEHV1 and EEHV2. However, after additional genetic analysis, those two cases have subsequently proven to be sufficiently distinct to be renamed as EEHV3 and EEHV4 (Richman et al., unpublished data). In both of those cases, two sets of degenerate universal herpesvirus PCR primers, including the TER set used originally to detect EEHV1 (Richman et al., 1999), and the Codehops POL primers used to detect numerous new herpesviruses of veterinary or wildlife interest (VanDevanter et al., 1996; Rose, 2005) each initially generated PCR DNA products from just one of four or five necropsy tissue samples available (colon from #NAP27 and heart from #NAP22). We then also designed a second generation set of more sensitive non-redundant EEHV3-specific TER primers and found that they readily detected the viral DNA by first-round PCR in all five tissue samples from case #NAP27 (EEHV3) and also from all four tissue samples from Case #NAP22 (EEHV4) (Garner et al., 2009).

In addition, a set of four different elephant gammaherpesviruses, which we refer to here as EGHVs, have also been reported in genital and conjunctival swabs of healthy captive elephants in the United States by Wellehan et al. (2008). Three of the latter were present in Asian elephants and the fourth in an African elephant. Ehlers et al. (2008) have also described an example of one of these same Asian elephant gammaherpesviruses that they termed EmaxGHV1.

Because of the large expansion in the number and genetic diversity of known elephant herpesviruses, we realized the necessity and value of developing several new sets of diagnostic PCR primers designed to improve the sensitivity and specificity for detection of a variety of distinct subsets of elephant herpesviruses. Here we describe a number of additional findings relating to the detection of both known and several previously unknown elephant herpesvirus genomes in either diseased or routine samples collected from captive Asian or African elephants that were forwarded to the National Elephant Herpesvirus Laboratory for analysis. The results presented include the first semi-quantitative measurements of viremia levels in any cases of elephant hemorrhagic
disease, the first demonstrations of the presence of EGHVs in localized lesions, nodules or blood samples, as opposed to in either genital or conjunctival swabs, as well as the first reports of multiple herpesvirus infections in a single elephant. In addition, we report the discovery of three more new elephant herpesvirus species, two in the A plus T-rich branch of the probosciviruses (EEHV5 and EEEHV6) and a fifth species of elephant gammaherpesvirus (EGHV5), which is most closely related to whale and dolphin gammaherpesviruses. Another new gammaherpesvirus referred to as EGHV3B is also reported from an African elephant, which is significantly different from EGHV3A found in Asian elephants, but these may not be sufficiently different to justify separate species status. This now brings the total number of known distinct species of elephant herpesviruses to 11 (or 13 if the two related sub-species pairs EEHV1A/EEHV1B and EGHV3A/EGHV3B are included). However, only the probosciviruses have been associated with acute systemic infection and microvascular damage in cases of fatal hemorrhagic disease, whereas none of the EGHVs have yet been suspected of contributing to systemic disease.

2. Materials and methods

2.1. EEHV and EGHV positive pathological samples

All examples of North American EEHV probosciviruses have been assigned a chronological NAP number. Those that are relevant to this paper are listed in Table 1. Note that the proboscivirus isolate numbers used match those of the EEHV disease Case Numbers from which they were obtained. The index case was Kumari (EEHV1 #NAP11) a captive-born female that died at 16 months of age at the National Zoological Park in Washington, DC in 1995. EEHV1A #NAP18 was obtained from a 2.5-year-old captive-born male Asian elephant that was born in Missouri and died in California in 2000. The index African elephant case EEHV2 #NAP12 occurred in a captive-born male calf that died at 11 months of age in California in 1996. The chimeric EEHV1B/1A #NAP19 virus was obtained from a 2.5-year-old captive-born female Asian calf that died at 11 months of age in California in 2003 and from case #NAP20, a 42-year-old, wild-born female African elephant that had vulval lesions in Tennessee in 2005. The novel gammaherpesvirus EGHV5 #NAG6 was found in a biopsy DNA sample taken from a papillomatous nodule present inside the trunk of a healthy 27-year-old wild-born male Asian elephant in Ohio in 2007. EGHV3A #NAG7 was found transiently at low levels in the blood of a 4-year-old male Asian calf with oral discoloration symptoms in Texas in 2009. The novel Proboscivirus genome EEHV5 #NAP28 was detected within two consecutive routine whole blood samples drawn two weeks apart from a living 59-year-old wild-born female Asian elephant without overt symptoms in Oct 2007. Another novel Proboscivirus genome EEHV6 #NAP35 was detected in a blood sample collected from a 15-month-old African elephant calf in Arkansas that survived after mild illness symptoms and treatment with famciclovir in Mar 2009. Positive EEHV1 control samples used include necropsy tissue DNA from case #NAP21 a 3-year-old female Asian elephant from New York State in 2003, and from case #NAP20, a 42-year-old, wild-born Asian elephant in Illinois in 2002.

2.2. PCR detection of elephant herpesvirus genomes

The templates for PCR amplification were total DNA samples purified from minced frozen necropsy tissue (Dako Medimachine, Cardinter, CA) or from whole blood using a Gentra Capture Column Kit (Genta Systems, Minneapolis, MN). The redundant POL PCR amplification employed the following conditions: 34 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min followed by 72 °C for 7 min, using Platinum PCR Supermix (Invitrogen, Carlsbad, CA). All other PCR amplification employed the

| Table 1 |
| Summary features of eleven elephant herpesviruses detected in these studies. |

<table>
<thead>
<tr>
<th>Virus name</th>
<th>Strain/case designation</th>
<th>Host animal Species, sex, age</th>
<th>Location</th>
<th>Pathology description</th>
<th>Tissue source</th>
<th>DNA size</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEHV3</td>
<td>#NAP27</td>
<td>EM, F, 6y</td>
<td>Washington</td>
<td>Fatality</td>
<td>Necropsy tissue (+ blood)</td>
<td>486-bp</td>
<td>EU568936</td>
</tr>
<tr>
<td>EGHV2</td>
<td>#NAG1</td>
<td>Same</td>
<td>Same</td>
<td>Same</td>
<td>Whole blood</td>
<td>271-bp</td>
<td>HM060766</td>
</tr>
<tr>
<td>EGHV4</td>
<td>#NAP22</td>
<td>EM, F, 5y</td>
<td>Oklahoma</td>
<td>Fatality</td>
<td>Necropsy tissue (+ blood)</td>
<td>499-bp</td>
<td>EU568934</td>
</tr>
<tr>
<td>EGHV3A</td>
<td>#NAG2</td>
<td>EM, F, 5y</td>
<td>Florida</td>
<td>Vulval lesions</td>
<td>Whole blood</td>
<td>171-bp</td>
<td>HM060767</td>
</tr>
<tr>
<td>EGHV2</td>
<td>#NAG4</td>
<td>Same</td>
<td>Same</td>
<td>Same</td>
<td>Same</td>
<td>271-bp</td>
<td>HM060769</td>
</tr>
<tr>
<td>EGHV3B</td>
<td>#NAG3</td>
<td>LA, F, 22y</td>
<td>Tennessee</td>
<td>Vulval lesions</td>
<td>Biopsy</td>
<td>457-bp</td>
<td>HM060768</td>
</tr>
<tr>
<td>EGHV2</td>
<td>#NAG5</td>
<td>Same</td>
<td>Same</td>
<td>Same</td>
<td>Same</td>
<td>271-bp</td>
<td>HM060770</td>
</tr>
<tr>
<td>EGHV3A</td>
<td>#NAG7</td>
<td>EM, M, 4y</td>
<td>Texas</td>
<td>Oral lesions</td>
<td>Whole Blood</td>
<td>172-bp</td>
<td>HM060772</td>
</tr>
<tr>
<td>EGHV5</td>
<td>#NAG6</td>
<td>EM, M, 27y</td>
<td>Ohio</td>
<td>Trunk nodule</td>
<td>Biopsy</td>
<td>457-bp</td>
<td>HM060771</td>
</tr>
<tr>
<td>EEHV5</td>
<td>#NAP28</td>
<td>EM, F, 60y</td>
<td>Wash, DC</td>
<td>Routine</td>
<td>Whole blood</td>
<td>463-bp</td>
<td>HM060764</td>
</tr>
<tr>
<td>EEHV6</td>
<td>#NAP35</td>
<td>LA, F, 15mth</td>
<td>Arkansas</td>
<td>Moribund</td>
<td>Whole blood</td>
<td>484-bp</td>
<td>HM060765</td>
</tr>
</tbody>
</table>

* Same case as above; EEHV, Elephant endotheliotropic herpesvirus; EGHV, Elephant gammaherpesvirus; NAP, North American Proboscivirus case/isolate #; NAG, North American Elephant Gammaherpesvirus case/isolate #; EM, Elephas maximus; LA, Loxodonta africana.

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2.3. DNA sequencing and phylogenetic analysis

All DNA sequencing was carried out by direct cycle sequencing on both strands of purified PCR DNA products from either one, two or three rounds of nested or seminested PCR amplification. The correct sized PCR products were purified after agarose gel electrophoresis with a Qiagen II Gel Extraction kit (Qiagen, Valencia, CA). Sequencing reactions were carried out with the ABI PRISM DigDye Terminator v3.1 cycle sequencing kit and analyzed with the AB 373A and 377A sequencers (Perkin Elmer, Norwalk, CT). Sequencing reactions were set up according to the manufacturer's instructions. The resulting DNA sequences obtained with the EEHV3/4-specific primers were both described previously (Garner et al., 2009). The virus Codehops DNA POL primer set (2595/2596/2597) was used to generate high efficiency TER primer set specific for EHV-3/4 and other herpesviral POL proteins in Genbank. Although the POL gene fragments differed from those of EHV1 and EHV2 by nearly 30% at the amino acid level, a G plus C-content of 67% and displayed no more than 55% identity to any other known herpesviral POL proteins in Genbank. Although the POL DNA sequences obtained from all five tissue samples from both cases and in an early disease blood sample from #NAP27 (day 4) taken four days before she succumbed (data not shown). As before, the resulting DNA sequences were analyzed using Assemblablig and Clustal-W nearest neighbor joining as implemented for MacVector vers 7, together with BLASTX or TBLASTX comparison programs provided online at NCBI.

3. Results

3.1. Further analysis of EEHV hemorrhagic disease cases with EEHV3/4-specific POL primers

Based on the successful large increase in sensitivity obtained with the EEHV3/4-specific primers, we set out here to design and test a set of first, second and third-round nested but non-redundant POL gene primers equivalent to the standard 480/520-bp Codehops region that would be expected to be both specific and sensitive for the EEHV4 and EEHV3 versions. Appropriate combinations of these new primer pairs 6719/7400/6720/6721 were positive by first-round PCR with a total of nine tissue samples tested from the fatal Asian elephant calf Cases #NAP27 and #NAP22, as well as from the first whole blood samples of both cases and in an early disease blood sample from #NAP27 (day 4) taken four days before she succumbed (data not shown). As before, the resulting DNA sequences of both of the EEHV3 and EEHV4 POL gene fragments differed from those of EHV1 and EHV2 by nearly 30% at the amino acid level, a G plus C-content of 67% and displayed no more than 55% identity to any other known herpesviral POL proteins in Genbank. Although the POL DNA sequences obtained from all five tissue samples from Case #NAP27 were identical to each other, and the same was true for all four tissue samples from Case #NAP22, the two sets differed at 38 nucleotides and by 16 amino acids (see Table 3). Because of the significant level of DNA and protein divergence between the two cases (at the U73/OBP

### Table 2

Listing of new EEHV DNA POL PCR primers used.

<table>
<thead>
<tr>
<th>Description</th>
<th>Number</th>
<th>Orient</th>
<th>Sequence (5'→3')</th>
<th>Pairing and product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific EEHV3/4:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. EB4P</td>
<td>A1</td>
<td>6719</td>
<td>R</td>
<td>-CGTGGAAAGTGTGCAGCAGTT-</td>
</tr>
<tr>
<td>2. EB4P</td>
<td>B1</td>
<td>7400</td>
<td>L</td>
<td>-CAGCATACATCGAGCGCTACTAC-</td>
</tr>
<tr>
<td>3. EB4P</td>
<td>B2</td>
<td>6720</td>
<td>L</td>
<td>-ATGTGCGGCGTGCCTGAC-</td>
</tr>
<tr>
<td>4. EB4P</td>
<td>B3</td>
<td>6721</td>
<td>L</td>
<td>-CTACACGCAAAGCCGCTCTA-</td>
</tr>
<tr>
<td>PAN EEHV and specific EEHV6:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. PAN-EBP</td>
<td>A1</td>
<td>6710</td>
<td>R</td>
<td>-ACAAACACCGTGTCRGRTRCVRCTA-</td>
</tr>
<tr>
<td>6. PAN-EBP</td>
<td>B1</td>
<td>6711</td>
<td>L</td>
<td>-GTCATTGATTGNCNNYYGTAYCC-</td>
</tr>
<tr>
<td>7. PAN-EBP</td>
<td>B2</td>
<td>6712</td>
<td>L</td>
<td>-TGVAAYGCGTNTYAYGGATTTACGG-</td>
</tr>
<tr>
<td>8. EB6P</td>
<td>A2</td>
<td>7584</td>
<td>R</td>
<td>-CTTAGCTTGGACCTCTCAGTC-</td>
</tr>
<tr>
<td>Specific EEHV5:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. EBSP</td>
<td>A1</td>
<td>7483</td>
<td>R</td>
<td>-CTACACGACAGCAGTACCTCC-</td>
</tr>
<tr>
<td>10. EBSP</td>
<td>B1</td>
<td>7484</td>
<td>L</td>
<td>-GTAATCATGTTACGGACACAGA-</td>
</tr>
<tr>
<td>11. EBSP</td>
<td>B2</td>
<td>7424</td>
<td>L</td>
<td>-CGACGCAACCTCATTTAACAC-</td>
</tr>
<tr>
<td>12. EBSP</td>
<td>B2</td>
<td>7485</td>
<td>L</td>
<td>-CGTCATATGATTACGGCAG-</td>
</tr>
<tr>
<td>PAN EGHV and specific EGHV2:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13. PANEGP</td>
<td>A1</td>
<td>6784</td>
<td>R</td>
<td>-GTGCGGACTTTGGCAGCTTACCC-</td>
</tr>
<tr>
<td>14. PANEGP</td>
<td>B1</td>
<td>6788</td>
<td>L</td>
<td>-ACMCNCTGTAATCCTGACAC-</td>
</tr>
<tr>
<td>15. PANEGP</td>
<td>A2</td>
<td>6785</td>
<td>R</td>
<td>-CCMCTGAYATTYWCAGGCCMCA-</td>
</tr>
<tr>
<td>16. EG2/3P</td>
<td>A3</td>
<td>6786</td>
<td>R</td>
<td>-CCCCTGATATACAGGCACAC-</td>
</tr>
<tr>
<td>17. EG2P</td>
<td>B3</td>
<td>6787</td>
<td>L</td>
<td>-ACCCCTGAAATCATACGATG-</td>
</tr>
<tr>
<td>PAN EGHV and specific EGHV3:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18. PANEGP</td>
<td>A1</td>
<td>6784</td>
<td>R</td>
<td>-GTGCGGACTTTGGCAGCTTACCC-</td>
</tr>
<tr>
<td>19. PANEGP</td>
<td>B4</td>
<td>7489</td>
<td>L</td>
<td>-GTCRTGTCTCCGCTAGGACAG-</td>
</tr>
<tr>
<td>20. PANEGP</td>
<td>A2</td>
<td>6785</td>
<td>R</td>
<td>-CCMCTGAYATTYWCAGGCCMCA-</td>
</tr>
<tr>
<td>21. EG3P</td>
<td>B5</td>
<td>7486</td>
<td>L</td>
<td>-CTCCCTGAAATCATACGAC-</td>
</tr>
</tbody>
</table>

following conditions: 95 °C for 2 min, then 45 cycles of 95 °C for 40 s, 50 °C for 45 s and 73 °C for 1 min following by 73 °C for 5 min (Promega, Madison, WI). The second generation high efficiency TER primer set specific for EHV3 (6707/6708/6727/6728) as well as the Pan Herpesvirus Codehops DNA POL primer set (2595/2596/2597) was both described previously (Garner et al., 2009). The descriptions and DNA sequences for six other new sets of EEHV DNA POL PCR primers designed specifically for these studies are listed in Table 2 together with the combinations used and sizes of the products of each primer pair.

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within their blood samples by limiting dilution PCR. In the
VETMIC-4919; No. of Pages 14

The latter result is very similar to that obtained
and 3125-fold after first and second-round PCR (data not
2/ 1.2 19/11.8 30/18.7 31/19.4 42/26 49/30.6
15/3.2 – 20/12.5 31/19.4 32/20 43/27 50/31.2
105/22 103/21.4 – 30/18.7 28/17.5 45/28 51/31.8
140/29 134/28 136/28.3 – 19/12 46/28.7 50/31.2
149/31 146/30.4 143/30 100/20.6 – 47/29.4 53/33
192/40 187/39 190/41.5 200/41.7 178/37 – 16/10
190/39.6 190/39.6 201/42 190/41.5 169/35.2 36/7.5 –

Amino acid and nucleotide differences (number/percentage)

1. EEHV1A – 53/33 59/37 59/37 59/37 65/40.6
2. EEHV1B 167/34.8 – 64/40 64/40 61/38 62/38.7
3. EEHV3 183/38 194/40.4 – 4/2.4 23/14.4 45/27
4. EEHV5 185/38.5 199/41.4 25/5.2 – 20/12.5 45/28
5. EEHV4 185/38.5 188/39 – 101/21.7 100/20.8 – 46/29
6. EGVH4 133/40.2 189/39.3 182/33.7 169/35.2 – –

Amino acid and nucleotide differences (number/percentage)

8. EGHV1 – 59/37 59/37 59/37 65/40.6
9. EGHV2 167/34.8 – 64/40 64/40 61/38 62/38.7
10. EGHV3A 183/38 194/40.4 – 4/2.4 23/14.4 45/27
11. EGHV3B 185/38.5 199/41.4 25/5.2 – 20/12.5 45/28
12. EGHV4 185/38.5 188/39 – 104/21.7 100/20.8 – 46/29
13. EGVH5 133/40.2 189/39.3 162/33.7 169/35.2 – –

Codehops region only (=480-bp, 160 amino acids from positions 605 to 777).

and U77/HEL gene loci as well as in U38 (POL), we have
derived the original designation (Garner et al., 2009) of
the virus in case #NAP27 from EEHV3A to EEHV3 and that
in Case #NAP22 from EEHV3B to EEHV4 (Richman, et al,
unpublished data). Importantly, additional semi-quantita-
tive comparisons with these two primer sets on diluted
samples revealed that, although they cross-reacted, the
EEHV4 TER primer set was about ten-fold more sensitive
for EEHV4 than for EEHV3. Both have been tested down to
five-fold dilutions for both, in which the total amount of
DNA present was maintained at a constant level by the
addition of human placental carrier DNA. In each case, the
original undiluted PCR reaction contained 2 µl of DNA
from a total 100 µl sample prepared from 200 µl of whole
blood. These diluted samples were then amplified by
nested PCR with our EEHV3-specific TER primer set (data
data not shown). The final #NAP27 (day 8) whole blood sample
proved to be positive results down to 625-fold and 15,625-fold dilution after first and second-round amplifi-
cation respectively, but was negative at the next dilution in
each case. A similar experiment was also carried out with
diluted #NAP22 whole blood using the EEHV4-specific POL
primer set, which gave positive results down to 125-fold
and 3125-fold after first and second-round PCR (data
data not shown). The latter result is very similar to that obtained
with whole blood samples from several untreated cases of
EEHV1 disease, which have been measured by quantitative
real-time PCR to have viral load values of close to 10^7 viral
gene copies per ml (Stanton et al., in press). Both primer
sets failed to amplify any EEHV3 or EEHV4 sequences (even
after third-round PCR) from both undiluted whole blood
DNA samples taken at the same time as the disease in Case
#NAP27 from all three herdmates, including one African
and two Asian elephants, as well as from a whole blood
sample collected one year prior to disease from the same
animal that died (data not shown).

3.2. Detection of an additional low level gammaherpesvirus
infection in the case of EEHV3-positive hemorrhagic disease

To assess whether any gammaherpesviruses might also
be present in elephant necropsy or blood samples, where
they could generate complications with regard to diagno-
sis, we next designed a set of redundant PAN EGHV primers
(6784/6785/6788). These were based on comparisons of
the available EGHV POL sequences with other mammalian
gammaherpesvirus POL DNA sequences, including elimi-
nating mismatches from both the outer and inner
Codehops primers. Using a combination of both first-
round moderately redundant consensus EGHV primers,
followed by a second-round with EGHV2-specific elephant
gammaherpesvirus primers (6786/6787), one of these
same gammaherpesvirus genomes (designated #NAG1)
was also detected within Case #NAP27, the same fatal
Asian elephant case that presented with high levels of the
Proboscivirus EEHV3. However, positive results for the
EGHV genome were only obtained after second-round PCR
(using 6785/6787) in undiluted liver, spleen, and intestine
samples, and also in the final whole blood sample (Fig. 1,
lanes 2, 5 and 6), but not in other necropsy tissues tested
such as colon and kidney (Fig. 1, lanes 3 and 4).

The unique 271-bp POL DNA sequence obtained for this
gammaherpesvirus proved to be identical to that previ-
ously described for EEH4 (Wellehan et al., 2008), which
we call EGHV2 to discriminate them from the EEHVs and to

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pathological samples from Asian and African elephants: Identification of two new probosciviruses (EEHV5 and EEHV6)
be consistent within the gammaherpesvirus set (see Table 4). No EGHV PCR products were detected in these samples after 5, 25 or 125-fold dilution. In contrast to Case #NAP27, a similar set of tests on all diluted and undiluted tissue DNA samples from Case #NAP22 were negative after two-round PCR with the same PAN EGHV primer set, except for a weak cross-reacting band from EEHV4 in the heart tissue sample only (Fig. 1, lane 9).

### 3.3. Detection of additional novel elephant herpesviruses with PAN EEHV primers

Because of the large increase in recognized elephant herpesvirus species, we next designed a set of two-round PAN-EEHV POL locus primers 6710/6711/6712, that both encompassed the known expanded sequence information about the EEHV1, EEHV2, EEHV3 and EEHV4 POL loci (Richman et al., unpublished data), and at the same time reduced the level of redundancy and mismatches compared to those in the standard Codehops PAN-herpesvirus primers. The new PAN-EEHV primers still proved capable of detecting both EEHV3 and EEHV4, as well as all other known EEHV positive samples tested by either first or second-round PCR within available positive necropsy tissue samples, including examples of EEHV1A, EEHV1B, EEHV2 and EEHV4 (Fig. 2, lanes 1–6). However, they were least efficient for EEHV3. This test has now been used over a three-year time period for routine screening of all blood, biopsy and necropsy samples submitted to the NZP Elephant Herpesvirus laboratory. In addition to being effective in detecting several new cases of EEHV1A or EEHV1B-associated hemorrhagic disease (not addressed

<table>
<thead>
<tr>
<th>Proposed name</th>
<th>ICTV number</th>
<th>Host species</th>
<th>Pathology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. EEHV1A</td>
<td></td>
<td>EM</td>
<td>Hemorrhagic disease</td>
<td>Richman et al. (1999)</td>
</tr>
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<td>2. EEHV1B</td>
<td></td>
<td>LA</td>
<td>Gastrointestinal hemorrhage</td>
<td>Fickel et al. (2001)</td>
</tr>
<tr>
<td>3. EEHV2</td>
<td></td>
<td>LA</td>
<td>Hemorrhagic disease</td>
<td>Richman et al. (1999)</td>
</tr>
<tr>
<td>4. EGHV1</td>
<td></td>
<td>LA</td>
<td>Gastrointestinal hemorrhage</td>
<td>Fickel et al. (2001)</td>
</tr>
<tr>
<td>5. EGHV2</td>
<td></td>
<td>EM</td>
<td>Mucosal shedding</td>
<td>Wellehan et al. (2008)</td>
</tr>
<tr>
<td>6. EGHV3A</td>
<td></td>
<td>EM</td>
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<td>Wellehan et al. (2008)</td>
</tr>
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<td>7. EGHV3B</td>
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<td>LA</td>
<td>Genital lesion</td>
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<td>8. EEHV4</td>
<td></td>
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<td>Wellehan et al. (2008)</td>
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<td>9. EEHV5</td>
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<td>EM</td>
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<td>Garner et al. (2009)</td>
</tr>
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<td>10. EEHV4</td>
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<td>EM</td>
<td>Hemorrhagic disease</td>
<td>Garner et al. (2009)</td>
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<tr>
<td>11. EEHV5</td>
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<td>EM</td>
<td>Mild viremia</td>
<td>This study</td>
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<tr>
<td>12. EGHV5</td>
<td></td>
<td>EM</td>
<td>Trunk nodule</td>
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</tr>
<tr>
<td>13. EEHV6</td>
<td></td>
<td>LA</td>
<td>Mild viremia</td>
<td>This study</td>
</tr>
</tbody>
</table>

*EM, Elephas maximus; LA, Loxodonta africana.
* Same case as above.

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We were unable to detect the other POL gene segment from either of these two EGHV2 viruses using the double PAN-EGHV primer set 6784/6788 at second-round (Fig. 3, lanes 7 and 8), nor were those primers able to detect the known high abundance EEHV1 genome present in a positive necropsy sample from Case #NAP20 (Fig. 3, lane 2).

3.5. Evidence for distinctive Asian and African forms of EGHV3

Introduction of another EGHV3-specific primer 7486 used together with the EGHV PAN primer 6785 for second-round PAN-EGHV POL primers. Lanes 1-5, detection of EGHV3 with first-round PAN-EGHV POL primers 6784/6788 followed by second-round EGHV3-specific primers 6785/6787 (310-bp); 6, Asian elephant #NAG6; 7, human placental DNA negative control (HPD); 4, Asian elephant #NAG4, vulval lesion; and 5, Asian elephant #NAG5, vulval lesion; and 8, African elephant #NAG2, vulval lesion. The three left-hand side lanes contain a 250-bp multimer size marker ladder.

Fig. 3. PCR detection of EGHV2 in an African and Asian elephant and EGHV5 in an Asian elephant.

Agarose gel electrophoretic separation of ethidium bromide-stained PCR products obtained using moderately redundant consensus PAN-EGHV or PAN-EEHV POL primers. Lanes 1 and 4, detection of EGHV2 with first-round PAN-EGHV primers 6784/6788 followed by second-round EGHV2-specific primers 6785/6787 (310-bp); 2, Asian elephant #NAP20 PAN-EGHV primer set 6785/6787 at second-round (Fig. 3, lanes 7 and 8), nor were those primers able to detect the known high abundance EEHV1 genome present in a positive necropsy sample from Case #NAP20 (Fig. 3, lane 2).

3.4. Detection of simultaneous infection with two different gammaherpesviruses in both an Asian and an African elephant with vulval lesions

Positive gammaherpesvirus POL PCR DNA products of 250-bp detected with our PAN-EEHV primers were identified during second-round PCR using 6710/6712 within blood obtained from a healthy 6-year-old captive-born female Asian elephant with vulval lesions (#NAG2), as well as directly in biopsy tissue from a healthy 22-year-old wild-born African elephant with vulval lesions (#NAG3) (data not shown). Despite the fact that we had intended these primers to preferentially detect all EEHV genomes, analysis of the unique 172-bp DNA sequences obtained here revealed instead two EGHV3 POL genes very similar to that of E1HV5 as reported in swabs from Asian elephants by Wellehan et al. (2008). The EGHV3 DNA sequence obtained from our Asian elephant blood sample (#NAG2) proved to be identical to that of the E1HV5 described by Wellehan et al. (2008), but there were 5 nucleotide differences here (2.9%) in the African elephant EGHV3 biopsy sample (#NAG3) compared to that of E1HV5.

Unexpectedly, in both animals with these vulval lesions, we also subsequently detected a second different gammaherpesvirus genome EGHV2 when we attempted to obtain the other segment of the 480-bp Codehops POL PCR locus using our new PAN-EEHV primer set. Both of the primer pairs 6784/6788 and 6785/6787 gave 310-bp bands after second-round PCR, in which one primer of each pair preferentially targets EGHV2 (Fig. 3, lanes 1 and 4). The Asian elephant EGHV2 (#NAG4) DNA sequence obtained here was again identical to that given for E1HV4 by Wellehan et al. (2008), but the African elephant EGHV2 (#NAG5) sample contained 3 out of 271-bp differences (1.1%) from the Asian version. We were unable to detect the other POL gene segment from either of these two EGHV2 viruses using the double PAN-EGHV primer set 6785/6787 at second-round (Fig. 3, lanes 7 and 8), nor were those primers able to detect the known high abundance EEHV1 genome present in a positive necropsy sample from Case #NAP20 (Fig. 3, lane 2).

here), we have also detected eight more examples of other elephant herpesviruses as described below, all within samples from healthy or surviving elephants, but which in most cases had some suspicious illness or observable lesions. Surprisingly, six of these positive samples proved to be elephant gammaherpesviruses, with five being variants of either the EGHV2 or EGHV3 viruses described by Wellehan et al. (2008), whereas the other represented a fifth different and novel species of elephant gammaherpesvirus (EGHV5). In addition, we also identified two more novel probosciviruses (EEHV5 and EEHV6) with these PAN EEHV primers.

E. Latimer et al./Veterinary Microbiology xxx (2010) xxx–xxx 7

Fig. 2. Detection of EEHV1A, EEHV1B, EEHV2 and EEHV4 with PAN EEHV POL primers.

Agarose gel electrophoretic separation of ethidium bromide-stained PCR products obtained using the moderately redundant PAN-EEHV POL Codehops region primers 6710/6711 (500-bp) then 6710/6712 (250-bp). The 250-bp ladders are shown on the left of both the center and right-hand panels. The following necropsy DNA samples were used. Lane 1, EEHV1B case #NAP4; 2, EEHV1A case #NAP11; 3, EEHV1A case #NAP17; 4, EGHV2 case #NAP12; 5, EEHV1B/1A case #NAP19; 6, EGHV4 case #NAP22 whole blood; 7, human placental DNA negative control (HPD). The samples from EEHV1B case #NAP11, EEHV1A case #NAP17 and EGHV4 case #NAP22 heart were also all strongly positive even after just first-round PCR with primers 6710/6711 (500-bp) not shown, whereas those from EEHV1A #NAP11 and EEHV1B/1A #NAP19 were only weakly positive first-round.

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round PCR after a first-round with 6784/7489 was successful in detecting the other 310-bp segment of the EGHV3 POL gene Codehops region from the African elephant vulval lesion biopsy (#NAG3) (data not shown). The combined 457-bp POL locus DNA sequence displayed 25 nucleotide (5.5%) and 5 amino acid (3.1%) differences from the prototype EGHV3 (E1HV5) POL gene described by Wellehan et al. (2008). Therefore, we have tentatively named the apparent African version of this virus as EGHV3B (#NAG3) to distinguish it from the closely related EGHV3A (#NAG2) virus found in both the blood from the Asian elephant with vulval lesions and in the Wellehan studies (see Table 4).

3.6. Transient appearance of EGHV3A in the blood from an Asian elephant calf with oral lesions

Another case occurred in a 4-year-old Asian calf (#NAG7) that was suspected of having early stage EEHV disease and was given pre-emptive famciclovir after displaying oral lesions and tongue discoloration. However, it proved instead to have a low level of EGHV3 present in a single blood sample taken before treatment (but not in samples collected after treatment). This time, a weak 250-bp PCR product band was detected after second-round amplification with the PAN EEHV primers 6710/6712, which proved to be identical to EGHV3A rather than an EEHV (data not shown). We were not able to amplify the other half of the POL codehops region from this sample. Oral lesions and tongue discoloration are often thought to accompany the initial stages of EEHV-associated disease, but this case illustrates that EGHVs need to be considered as well as likely causes of non-hemorrhagic oral lesions.

3.7. Identification of a novel elephant gammaherpesvirus (EGHV5) in a trunk growth

A third different elephant gammaherpesvirus was also detected with our PAN EEHV primer set 6710/6711/6712 after two-round PCR in a biopsy DNA sample obtained from a proliferating papilloma-like growth present inside the trunk of a healthy wild-born 27-year-old male Asian elephant (#NAG6; data not shown). The initial second-round 250-bp DNA sequence obtained from this sample proved to be that of a novel DNA POL gene that clearly fits within the gammaherpesvirus subfamily, but is phylogenetically closer to the POL proteins of bottlenose dolphin gammaherpesvirus and of beaked whale gammaherpesvirus than to any other known herpesviruses. Notably, it is also significantly closer to both of those virus species than to any of the other four known elephant gammaherpesviruses (see Fig. 6B). Pathological examination of the papilloma revealed the presence of typical herpesvirus nuclear inclusion bodies and virions within differentiated epithelial cells.

This novel EGHV genome represented the ninth EHV genome that we have identified in Asian and African elephants: Identification of two new probosciviruses (EEHV5 and EEHV6) and two new gammaherpesviruses (EGHV3B and EGHV5). Vet. Microbiol. (2010), doi: 10.1016/j.vetmic.2010.05.042

A fourth example of an elephant herpesvirus (isolate #NAP28) identified with our PAN EEHV primers 6710/6711/6712 was found when carrying out routine PCR virus blood typing on the sample from the 59-year-old wild-born female Asian elephant, that had shown signs of mild illness nine months earlier and occasionally displayed small oral lesions. Initially, 250-bp second-round PCR products (data not shown) were obtained with the 6710/6712 primers from two sequential blood samples collected over a two-week period, but not from a third at one month later. Evaluation of the DNA sequence of these two PCR products revealed identical but novel POL genes that were more closely related to the EEHVs than to any other known herpesviruses, but were also distinctly different from all other previously identified EEHV POL genes. Therefore, we have designated this new elephant herpesvirus as EGHV5.

The EGHV5 viral DNA was of low abundance and difficult to detect consistently even after second-round PCR with the PAN EEHV primers. Therefore, we designed second-generation EEHV5-specific PCR primers and successfully amplified the known EGHV5 POL region to the more standard 480-bp Codehop region size, to permit more robust phylogenetic analysis. Two alternative second-round primers pairs from the final chosen primer set of 7483/7484/7424/7485 produced strong PCR bands of 480-bp and 230-bp from both the first and second week blood samples from #NAP28 (Fig. 4, lanes 1, 2, 4 and 5); however, again the one month later blood sample from the same animal was negative (lanes 3 and 6). The combination of data from the two PCR products yielded a 499-bp DNA sequence with a G plus C-content of 43% similar to that of EEHV1 and EEHV2, but with 19 (12%) and 31 (19%) amino acid differences from those of the orthologous segments of the EGHV1, EGHV2, EGHV3 and EGHV4 POL proteins, respectively (Table 3). Subsequent evaluation with these same specific primers of 24 sequential routine blood samples drawn from this elephant over the previous two-years proved to be negative (data not shown) implying that this
animal had undergone a transient but low-level asymptomatic systemic reactivation event.

3.9. Identification of EEHV6: a novel Proboscivirus that is most closely related to EEHV1

Finally, evidence for yet another previously undetected proboscivirus was obtained by PCR with the PAN EEHV primers on a blood sample collected from a 15-month-old female African elephant calf with limb stiffness (a commonly encountered symptom in other EEHV cases). This animal was treated prophylactically with famciclovir.

The sources, virus/case numbers and species identification of all eleven examples of elephant herpesviruses infections reported on and studied here are listed in the summary in Table 1, together with their Codehops region DNA POL sequence Genbank accession numbers. A predicted protein comparison over the 76-amino acid right-hand side of the POL Codehops region is shown in Fig. 5 for all 13 known types of elephant herpesviruses, including the two sub-species pairs EEHV1A/1B and EGHV3A/3B.

3.10. Phylogenetic comparison of all EEHV and EGHV DNA POL proteins

The sequences showed 40% G plus C content with an encoded DNA POL protein segment that differs from the EEHV1A, EEHV1B, EEHV5, EEHV2, EEHV3 and EEHV4 versions by 19, 20, 28, 30, 45 and 51 amino acids respectively (Table 3). Therefore, this new virus, which we have termed EEHV6, displays close to the same level of overall genetic divergence from EEHV1 as do EEHV5 from EEHV2 and EEHV4 from EEHV3. Because of the very young age of this calf and the fact that both Asian and African elephants were housed at this facility, we cannot make any interpretations as yet about whether EEHV6 is most likely to be endogenous to either African or Asian elephants. Clearly, it is also not known at this stage whether EEHV6 has the potential to cause severe pathogenesis in African elephants or not.
The predicted amino acids from codon positions equivalent to 702-777 of the EEHV1B (Kiba) DNA polymerase gene (Ehlers et al., 2006) are shown. The upper seven lines in the diagram represent the members of the Proboscivirus genus or EEHVs, whereas the lower six lines represent the elephant gammaherpesviruses or EGHVs. Conserved amino acids across both groups are shown in red, whereas amino acids that are specific to all EEHVs are shown in black. Similarly, common amino acids that are unique to all of the elephant gammaherpesviruses are shown in grey and those that are unique to individual virus species within the elephant gammaherpesviruses are shown in black. Signature amino acids that are characteristic for EEHV1, EEHV4 and EGHV5 are overlined or underlined. Note that although EEHV1A and EEHV1B have an identical amino acid structure in this region of POL their DNA sequences differ at eight nucleotide positions here and several other proteins encoded by the chimeric EEHV1B genome differ from those of EEHV1A by between 8 and 40% at the amino acid level (Richman et al, unpublished data).

Fig. 5. Comparison of protein sequences within a 76 amino acid Codehops segment of the DNA POL genes of all 13 known elephant herpesvirus species or sub-species.

when an alternative method of phylogenetic analysis was used (UPGMA, data not shown). The probosciviruses evidently separated from all other herpesvirus groups very early in Betaherpesvirinae evolution and diverged into multiple subgroups with major branching events estimated to be of the order of 35, 20 and 10 million years ago.

4. Discussion

Until very recently, the three probosciviruses EEHV1A, EEHV1B and EEHV2 that were identified originally as the causes of fatal elephant hemorrhagic disease (Richman et al., 1999; Fickel et al., 2003) were the only known elephant herpesviruses. Therefore, it was considered sufficient to use just the original PCR primer sets that were designed specifically for small segments of the TER or POL herpesvirus primers, we described a set of highly sensitive second-generation TER primers that are specific for EEHV3 plus EEHV4 and used them for preliminary genetic evaluation of these two novel viruses (Garner et al., 2009). However, the DNA POL gene locus is more variable than TER and is more commonly used in pathological studies. It also offers a more precise and reliable indicator of phylogenetic relationships amongst herpesviruses. Therefore, we needed to also develop a matching set of EEHV3 and EEHV4-specific POL primers as described here.

In addition, to encompass the possibility of there being more as yet undiscovered elephant herpesviruses both within the Proboscivirus genus (EEHVs) and amongst the newly described elephant gammaherpesviruses or EGHVs (Wellehan et al., 2008), we also developed two sets of moderately redundant POL locus PCR primers focused on broad range detection of each of these two classes of elephant herpesviruses. Our rationalization that these would likely be valuable diagnostic reagents with regard to
understanding and controlling elephant herpesvirus-associated disease has been amply confirmed by the subsequent rapid identification by PCR sequencing procedures of eleven more examples of elephant herpesviruses (other than EEHV1) within blood, lesions or nodules of healthy and diseased Asian and African elephants. These include \(10^7\) viral genome copies per ml. Therefore, considering that the highest viral loads for EEHV3 and EEHV4 being \(10^7\) viral genome copies per ml or greater, make a compelling case that these two viruses were indeed the primary etiological agents responsible for the hemorrhagic endothelial cell disease and deaths of these two juvenile Asian elephants. We emphasize that our original detection of EEHV3 and EEHV4 with the standard PAN mammalian herpesvirus PCR primers was highly serendipitous and was successful only because of the extraordinarily high viral load in just one tissue sample from each case. These viruses have proven to be so diverged in their DNA POL sequences that they each have four to five nucleotide mismatches with one or more of the highly redundant generic primers. In fact, both the standard PAN-herpes TER and POL redundant primers all gave negative results on first and second-round PCR in the NAP#27 and NAP#22 blood samples, despite the high viral load there, compared to with the later EEHV3/4-specific viral endothelial cells, our semi-quantitative estimates of viral loads for EEHV3 and EEHV4 being \(10^7\) viral genome copies per ml or greater, make a compelling case that these two viruses were indeed the primary etiological agents responsible for the hemorrhagic endothelial cell disease and deaths of these two juvenile Asian elephants. We emphasize that our original detection of EEHV3 and EEHV4 with the standard PAN mammalian herpesvirus PCR primers was highly serendipitous and was successful only because of the extraordinarily high viral load in just one tissue sample from each case. These viruses have proven to be so diverged in their DNA POL sequences that they each have four to five nucleotide mismatches with one or more of the highly redundant generic primers. In fact, both the standard PAN-herpes TER and POL redundant primers all gave negative results on first and second-round PCR in the NAP#27 and NAP#22 blood samples, despite the high viral load there, compared to with the later EEHV3/4-specific
primers, which improved the sensitivity by two orders of magnitude.

Wellehan et al. (2008) described four distinct species of gammaherpesviruses detected by PCR in conjunctival or genital swabs from 11 of 21 tested healthy elephants in captivity. These included three sequenced POL DNA fragments referred to by them as EEHV3, EEHV4 and EEHV5 in Asian elephants and another EEHV6 in the single African elephant tested. Some of the positive Asian elephant swabs were taken from lymphoid vulvitis conditions that have been described as being very common in both Asian and African elephants (Munson, 1995). To clearly distinguish this class of elephant herpesviruses from the proboscivirus, we and others (Ehlers et al., 2008) have proposed to instead name and number them as elephant gammaherpesviruses (e.g. EGHV1, EGHV2, EGHV3 and EGHV4, respectively). The four new probosciviruses EEHV3, EEHV4, EEHV5 and EEHV6 as well as the new fifth elephant gammaherpesvirus EGHV5 now represent the seventh, eighth, ninth, tenth and eleventh distinct species of herpesviruses found amongst either Asian or African elephants, which would likely be named EEHV7 to EEHV11 under the current formal ICTV nomenclature (Table 4).

More extensive genetic analysis to be reported elsewhere (Richman et al., unpublished data) confirms that all six probosciviruses differ significantly at multiple additional genetic loci, such as the U73/Origin Binding Protein and U77/Helicase genes as well as in the U38/DNA POL gene, and that EEHV3 and EEHV4, EEHV2 and EEHV5, and EEHV1 and EEHV6 tend to cluster together into three paired sister clades in phylogenetic trees at all of these loci. Furthermore, over a larger scale, the EEHV1 and EEHV2 genomes, whilst essentially colinear, diverge by an average of 20% at the protein level over all 30 gene loci examined across an 85-kb central segment of their expected to be 200-kb genomes. In addition, the EEHV1 viral genomes are themselves divisible into two distinct subtypes called EEHV1A and EEHV1B that are complex chimeric mosaics relative to one another. Including the EGHV3A/EGHV3B variant pair described here, the EEHV1A/EEHV1B pair makes a total of thirteen distinct types of herpesviruses altogether that are now known to infect Asian and/or African elephants (Table 4). As illustrated in these studies, maximal efficiency and accurate detection of these numerous elephant herpesviruses when screening pathological samples requires the use of a complex series of both partially redundant generic and highly specific non-redundant PCR primer sets, as well as direct DNA sequencing of all PCR products obtained.

Infections with the EGHVs within captive elephant populations have apparently remained ubiquitous and largely asymptomatic. In general, gammaherpesviruses are thought to persist at a low level in the latent state primarily in lymphoid cells, although their frequent detection in genital mucosal and conjunctival secretions of elephants as described by Wellehan et al. (2008) implies that reactivation may be common in these hosts. We estimate that the levels of EGHV2 (#NAG1) DNA present in the blood of Case #NAP27 were at least 5000-fold lower than the very high levels of EEHV3 (#NAP27) DNA carried there. Nevertheless, this result represents the first example of detection of any elephant gammaherpesvirus associated with systemic infection within blood or internal organs, where it was most likely a reactivated passenger accompanying a primary infection by the pathogenic EEHV3. Although no EGHV2 was detected within the acute disease tissue or blood samples from Case #NAP22, our experiments did not address the possibility of other EGHVs being present at similar low levels.

So far, neither the EEHV1A, EEHV2, EEHV3 nor EEHV4 viruses have ever been found at high enough levels for diagnostic first or second-round PCR detection within routine blood samples from any of the numerous healthy captive Asian elephants that have undergone testing, nor in unrelated necropsy tissue samples. Rather (with a single recent exception involving EEHV1B) they have all only been found in association with cases of acute systemic disease, which all included a high level of viremia as well as microvascular endothelial cell damage in most major organs in necropsy tissue (Richman et al., 2000a; Garner et al., 2009). However, new tests with the more sensitive second-generation primer sets and by real-time PCR have begun to detect low levels of virus in blood at early stages of viremia as well as in trunk washes of healthy herdmates, suggesting that (like the EGHVs) latent infection and very low level localized reactivation and shedding by EEHV1 at least may also be quite common (Stanton et al., in press).

The clearly implied causal association of EGHV5 with a proliferating mucosal epithelial papilloma also represents a novel finding and should generate caution about automatically assuming that all such abnormal growths, including those containing inclusion bodies, are necessarily associated with the pathogenic EGHVs. On the other hand, the two examples of both EGHV2 (#NAG2 and #NAG3) and EGHV3 (#NAG4 and #NAG5) detected in biopsied vulval lesions or blood of otherwise asymptomatic captive elephants may be similar to the observations using genital secretion swabs by Wellehan et al. (2008), and do not necessarily imply that they were directly causative of the biopsied lesions themselves. Furthermore, one of our two cases with genital lesions carrying both EEHV2 and EEHV3 was an African elephant. Both of these two virus species were recognized only within Asian elephant swab samples by Wellehan et al. (2008), but our wild-born African sample contained significantly diverged versions of both viruses, especially that of EGHV3B, which we judge to be sufficiently different to warrant being named as a distinctive variant compared to EGHV3A. Although this is a complex issue, the differences found between the African versus Asian elephant versions of EEHV2 and EGHV3 may imply that there has not (yet) been much intermingling of elephant host-specific versions of the gammaherpesviruses in captivity. It also raises the question of whether there may well be additional distinct African and Asian elephant versions of some of the other gammaherpesviruses and even of the probosciviruses that have not yet been identified.

In contrast to the EGHVs, infections with EEHVs can sometimes be highly pathogenic, especially in juvenile Elephas maximus. EEHV1A, EEHV1B, EEHV2, EEHV3 and EEHV4, but not yet EEHV5 or EEHV6, have all been found associated with cases of fatal hemorrhagic disease. The
phyllogenetic trees show that the probosciviruses have evidently co-evolved with their natural hosts the Elephants throughout the past 100 million years since they separated from all other mammalian groups. Therefore, infections with these viruses would be expected to be well-adapted to and universal in modern elephants, as well as capable of persisting in a long-term latent state in the wild within their natural host species. However, instead of the normal asymptomatic primary infections as infants, something as yet unknown about the situation (with EEHV1 especially) in captive juvenile Asian elephants leads to an inability of the victims to cope with or control what we presume to be primary infections. The key factors involved here could include some or all of the following: (1) far later than normal primary infections; (2) the absence of maternal antibodies or of potentially protective latent infections with other members of the EEHV group; and (3) being infected with the wrong species or subspecies of EEHV, which have not become evolutionarily adapted to a particular Asian elephant host species or subspecies. This latter situation may parallel that of the Rhesus B-virus (Macaque herpesvirus 1), an alphaherpesvirus that is asymptomatic in its natural host species, but has high lethality in the rare occasions in which it is transmitted to humans (Elmore and Eberle, 2008).

Other than the sometimes successful treatment with the anti-herpesvirus drugs famciclovir or ganciclovir (Schmitt et al., 2000), the best long-term solution to this devastating disease will most likely be the development of adapted attenuated live or killed EEHV vaccines. However, the inability as yet to be able to grow any of these viruses in cell culture, together with the now very large number of different herpesviruses identified, greatly complicates the situation. Attempts to obtain additional genomic DNA sequence data for more robust comparative phylogenetic analyses between the most closely related proboscivirus pairs EEHV3 and EEHV4, or EEHV2 and EEHV5, as well as EEHV1 and EEHV6 are in progress. The PCR reagents described here should make possible the detection of both additional virus species and as yet unidentified cases of this disease, as well as set the stage for efforts to try to definitively deduce the natural source host species for each of the pathogenic probosciviruses.

5. Conclusions

Five new PCR primer sets were designed to be: (1) specific for the EEHV3 plus EEHV4 POL gene; (2) generic for all known EEHV POL genes; (3) Generic for all known EGHV POL genes; (4) specific for EEHV5 and (5) specific for EEHV6. In addition to being used to diagnose or confirm several additional new cases of EEHV1-associated acute disease (to be addressed elsewhere), the generic primer sets were effective in detecting numerous examples of elephant herpesviruses other than EEHV1A or EEHV1B in either routine or pathological samples from Asian and African elephants. Based on initial DNA sequence data from the generic primers, we then generated and tested more sensitive specific primers for each of the new viruses and used them to evaluate virus loads and extend the genetic comparisons of all of these species over the full 480-bp Codehops segment of the DNA polymerase PCR locus.

Specifically, we have shown here that: (a) the new EEHV3/4 POL primers are capable of detecting both EEHV3 and EEHV4 in all necropsy tissue samples of the two lethal cases; (b) there were extremely high levels of viremia reaching at least 10^7 viral genomes per ml in the blood of the lethal EEHV3 and EEHV4 cases; (c) low levels of the known elephant gammaherpesvirus EGHV2 and EGHV3 were detected in blood from two Asian elephants, one being the fatal case of EEHV3 disease and the other having oral lesions; (d) coinfection with both EGHV2 and EGHV3 was detected in two elephants with vulval lesions, in one case from the blood of an Asian elephant and in the second case directly within a biopsy from an African elephant; (e) the PCR DNA sequence data here revealed apparent Asian (EGHV3A) and African (EGHV3B) specific variants of EGHV3; (f) a fifth elephant gammaherpesvirus named EGHV5 was identified in a mucosal trunk papilloma biopsy from a healthy captive Asian elephant; (g) a fifth distinct proboscivirus named EEHV5 was also identified in routine blood samples from a healthy adult Asian elephant; and finally (h) another novel proboscivirus named EEHV6 was identified in blood from an ailing African elephant calf.

Clearly, both Asian and African elephants naturally harbor multiple species of herpesvirusus from at least two distinct sub-families: the Proboscivirus genus within the betaherpesviruses and a diverged group of gammaherpesviruses. As expected, these viruses have complex interactions with their elephant hosts, which may include potential cross-species infections, and unfortunately with the EEHVs this can lead to a lethal hemorrhagic disease in calves that is currently a major worldwide problem for successful breeding programs for endangered Asian elephants. Extensive surveys to search for other potential examples of EEHV3, EEHV4, EEHV5, EEHV6 or EGHV5 within captive or wild Asian and African elephants would likely be highly informative.

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E. Latimer et al./Veterinary Microbiology xxx (2010) xxx–xxx


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