Short communication

Development of a SYBR Green I-based real-time PCR for detection of elephant endotheliotropic herpesvirus 1 infection in Asian elephants (Elephas maximus)

Ladawan Sariya, Jarin Chatsirivech, Parut Suksai, Witthawat Wiriyarat, Adisak Songjaeng, Siriporn Tangsudjai, Oraphan Kanthesaawee, Umaporn Maikaew, Kritsada Chaichoung

A B S T R A C T

Elephant endotheliotropic herpesvirus 1 (EEHV1) can cause fatal hemorrhagic disease in Asian elephants (Elephas maximus). Several studies have described this virus as a major threat to young Asian elephants. A SYBR Green I-based real-time polymerase chain reaction (PCR) was developed to identify EEHV1 on trunk swabs and necropsied tissues. Two of 29 (6.9%) trunk swab samples from healthy Asian elephants were positive for EEHV1. The viruses were analyzed and classified as EEHV1A based on 231 nucleotides of the terminase gene. Necropsied spleen and heart tissue showed the highest level and second highest levels of DNA virus copy accumulation, respectively. The detection limit of the test was 276 copies/μl of DNA. There was no cross-reaction with other mammalian herpesviruses, such as herpes simplex virus 1 and equine herpesvirus 2. Inter- and intra-assay showed low coefficients of variation values indicating the reproducibility of the test. The results indicated that the test can be practically used for epidemiological study, clinical diagnosis, and management and control of EEHV1.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Elephant endotheliotropic herpesvirus 1 (EEHV1) is a member of the genus Proboscivirus in the subfamily Beta herpesvirinae (Davison et al., 2009) and has been classified into subtype, EEHV1A and EEHV1B (Ehlers et al., 2006; Fickel et al., 2001). The virus is associated with cases of fatal hemorrhagic disease in Asian elephants (Elephas maximus) and also has been found in skin nodules and vesicular lymphoid patches of healthy African elephants (Loxodonta africana) (Richman et al., 1999, 2000). It has been proposed that EEHV1 can spread from otherwise healthy African elephants to Asian elephants within captivity (Richman et al., 1999, 2000; Ryan et al., 2001). The virus targets preferentially elephants between the ages of 4 months and 15 years and causes considerable loss of captive elephants in zoological gardens in Asia (Reid et al., 2006). Recently, EEHV1 has been detected in healthy Asian elephants (Hardman et al., 2011; Schafenaar et al., 2010; Stanton et al., 2010). Until now, EEHV could not be cultured from infected elephants with endothelial disease (Latimer et al., 2011; Ossent et al., 1990; Richman et al., 1999). Hence, virus detection in exposed elephants was attempted mostly by viral genome detection by polymerase chain reaction (PCR) based methods and serological assay for antibody detection. There have been several reports on PCR developed to detect viral DNA in whole blood samples, post-mortem necropsied tissues, and trunk washes (Fickel et al., 2001, 2003; Garner et al., 2009; Hardman et al., 2011; Latimer et al., 2011; Stanton et al., 2010). However, a new test that has exceeded the sensitivity of these methods is available currently. This study aimed to develop the SYBR Green I-based real-time PCR assay for detecting EEHV1 by the specified terminase gene.

2. Materials and methods

2.1. Design of primers

The nucleotide sequences of the terminase gene of EEHV1 available in the GenBank database were aligned using BioEdit version 7.0.4 software. Primers were selected from conserved regions and designed as EEHV-term-F: 5′-CTG ATA TCC TAC GTA TGT GAA GAA CAC-3′ and EmEEHV2: 5′-GTG TGC GCT AAA TGT TCT CTG-3′. The size of PCR products generated from these primers was 286 bps.
2.2. Preparation of standard plasmid

The terminase gene was amplified by primers EEHV-term-F and EmEEHV2 using the Expand High Fidelity PCR System (Roche, Mannheim, Germany), according to the manufacturer’s instructions. The amplification was initiated by one cycle of 95 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, and one additional extension at 72 °C for 7 min. After amplification, the PCR products were purified using the QiAQuick® Gel Extraction Kit (QIAGEN, Hilden, Germany), ligated into the pGEM®-T Easy Vector (Promega, Madison, USA) and transformed by One shot® TOP10 Chemically Competent Escherichia coli (Invitrogen™, CA, USA). The recombine plasmid was purified using the QiAprep® Spin Miniprep Kit (QIAGEN, Hilden, Germany) with a concentration measured at 260 nm to calculate the DNA copy number. The plasmid was kept at −80 °C until use.

2.3. Establishment of real-time SYBR Green I-based PCR

The real-time SYBR Green I-based PCR amplification was performed at a total volume of 25 μl. The reaction mixture contained 12.5 μl of 2xQuantiTect® SYBR® Green PCR (QIAGEN, Hilden, Germany), 0.3 μl of each primer, and 1 μl of standard DNA. The reactions were carried out in the Chromo4™ real-time PCR instrument (BIO-RAD, CA, USA) under the following conditions: one cycle of 15 min at 95 °C to activate DNA polymerase, followed by 40 cycles of 15 s at 94 °C, 30 s at 56 °C, and 30 s at 72 °C. After PCR cycling, the amplified PCR products were performed melting curve analysis to measure the specificity of PCR and to confirm their identity by the specific melting temperature (Tm) profiles. The melting curve was conducted at 50–95 °C by increasing the temperature by 0.2 °C/s with continuous fluorescence measurement. Tm of the amplified PCR product was calculated by the PCR instrument’s software.

2.4. Sensitivity, specificity, and reproducibility of the SYBR Green I-based real-time PCR

To determine the detection limit of the assay, standard plasmid was used as a template and was 10-fold serially diluted with nuclease-free water, giving 2.76 × 10^7–2.76 × 10^5 copies/μl of DNA. Each dilution was performed in triplicate and simultaneously operated by real-time PCR.

The specificity of the real-time PCR was determined using DNA samples of other mammal herpesviruses including herpes simplex virus 1 (HSV1) and equine herpesvirus 2 (EHHV2), along with a negative control (genomic DNA from a healthy elephant), a no template control using nuclease-free water (NTC), and a positive control (EEHV1 DNA: GenBank accession no. JF742644), and processed with the real-time PCR under the same conditions.

To determine the reproducibility of the test, standard plasmid DNA was diluted in concentrations ranging from 2.76 × 10^7 to 2.76 × 10^5 copies/μl and real-time PCR was performed. Each dilution was repeated three times (intra-assay) and processed three different times (inter-assay). Coefficients of variation (CV) values for the threshold cycle (Ct) were determined in both assays.

2.5. EEHV1 detection in the samples

This study was approved by the Faculty of Veterinary Science-Animal Care and Use Committee (protocol no. MUAVS-2010-20), Mahidol University. Necropsied tissues including tongue, lung, heart, kidney, ovary, lymph node, spleen, and liver, as well as blood samples, were collected from the carcass of a 5-year-old Asian elephant with suspected EEHV infection. DNA extraction was performed on each clinical specimen (10–20 mg of necropsied tissue and 200 μl of blood) using the DNeasy® Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to instructions from the supplier.

In addition, trunk swabs from 29 healthy Asian elephants were also collected. The swabs were stored in 15 ml centrifuge tubes containing 3 ml of viral transport medium (VTM) which was Eagle’s minimum essential medium with Hanks’ salts, supplemented with 0.5% gelatin and antibiotics, and transported at 4 °C to laboratory within 24–48 h. In laboratory, the VTM tubes containing the swabs were stirred and the swab was removed. The tubes were centrifuged at 3500 × g for 10 min at 4 °C. After this process, all but 200 μl of the supernatant was discarded. The pellet was resuspended in the remaining solution and DNA was extracted using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany). The DNA samples, standard plasmid DNA, and NTC were subjected to the real-time PCR. The amount of DNA copied in the positive samples was calculated by copy/g of tissue or copy/ml of blood or swab solution.

The quality of extracted DNA of the samples that were negative for EEHV1 (including other mammal herpesviruses) was further determined by amplifying the housekeeping gene β-actin, with primers β-actin-F: 5′-CGG GAC CTG ACT GAC TAC CTC-3′ and β-actin-R: 5′-CTT TAA TTA CAC CCA CGA TTT CC-3′, using real-time SYBR Green I-based PCR amplification. The PCR for β-actin amplification was performed under the same conditions as those for EEHV1 detection.

2.6. DNA sequencing and phylogenetic analysis

The samples that were positive for EEHV1 were amplified further using primers EmEEHV1 (5′-GTA CGT CCT TTC TAG CTC AC-3′) and EmEEHV2 for DNA sequencing. The conditions for these primers were carried out as the PCR conditions for standard plasmid preparation described previously. The PCR products from amplification were 337 bps. The sequencing data was analyzed by BLAST program. An unrooted phylogenetic tree using 231 nucleotides of terminase gene sequences of EEHV1–4 was constructed using the ClustalX program (v 1.8) and the PHYLIP package (v 3.65).

3. Results

3.1. Sensitivity and specificity of real-time SYBR Green I-based PCR assay

Various concentrations of standard plasmid were used to generate a standard plasmid curve. A strong linear curve was observed in the dilutions between 2.76 × 10^2 and 2.76 × 10^7 copies/μl of DNA with correlation coefficient (r^2) values equal to 0.999 (Fig. 1A). Quantitative data showed that the detection limit of the real-time PCR was 276 copies/μl of DNA (Fig. 1B).

A comparative study including EEHV1 (from positive sample), HSV1, EHHV2, the positive control, negative control, and NTC was performed. The results showed that only EEHV1 and the positive control were positive for EEHV1 (Fig. 2A). To verify that only the intended target was amplified, the melting curve was analyzed. The result demonstrated the Tm of amplified PCR products from EEHV1 was similar to that of the positive control and that no other PCR products were amplified (Fig. 2B). These results indicate the test has a sufficient level of specificity.

3.2. Reproducibility of the real time SYBR Green I-based PCR

The reproducibility of the assay was demonstrated using triplicates of each dilution of standard plasmid and then performing the real-time PCR. CV values of triplicates of intra- and inter-assay were relatively small (less than 3%), and ranged between 1.32% and 0.32%.
Fig. 1. A standard curve of standard plasmid in the SYBR Green I-based real-time PCR assay. (A) Standard curve was generated by plotting the mean Ct value of the replicate standards (x-axis) versus log quantity of standard (y-axis), with the error bar representing the standard deviation. The standard curve is linear in the dilutions from \(2.76 \times 10^2\) to \(2.76 \times 10^7\) copies/μl of DNA, with \(r^2\) and the slope values of the regression curve \(y = -0.2326x + 10.56\) and \(r^2 = 0.999\), respectively. (B) The intensity of standard curve.

3.3. Detection of the clinical samples by real-time PCR

Real-time SYBR Green I-based PCR was used simultaneously with eight organ tissue samples and one blood sample that were collected from an Asian elephant with suspected EEHV infection. Table 2 shows the real-time PCR was able to detect EEHV1 in four of the nine samples. The virus accumulated in spleen, heart, and tongue tissue, and whole blood, in decreasing order. The test was also able to detect EEHV1 in trunk swabs of healthy Asian elephants. Two (no. EEHV11/54 and EEHV28/54) of the 29 trunk swab samples (6.90%) were positive. For negative samples, β-actin
Fig. 2. The other herpesviruses (HSV1 and EHV2), EHHV1, positive control (Pos), negative control (Neg), and NTC were simultaneously operated by real time SYBR Green I-based PCR to determine the specificity of the test. (A) Data graph. The data graph was plotted as the fluorescence intensity at each cycle. (B) Melting curve. The curve was generated by plotting the negative first derivative of the fluorescence intensity versus temperature (−dI/dT).

Table 1
The reproducibility of real-time SYBR Green I-based PCR for EHHV1 terminase gene detection.

<table>
<thead>
<tr>
<th>Standard plasmid concentration (copies/μl of DNA)</th>
<th>n</th>
<th>Intra assay</th>
<th>Inter assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Avg. C&lt;sub&gt;T&lt;/sub&gt;</td>
<td>SD</td>
</tr>
<tr>
<td>2.76 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>3</td>
<td>34.85</td>
<td>0.44</td>
</tr>
<tr>
<td>2.76 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>3</td>
<td>30.99</td>
<td>0.14</td>
</tr>
<tr>
<td>2.76 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3</td>
<td>26.18</td>
<td>0.19</td>
</tr>
<tr>
<td>2.76 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>3</td>
<td>21.66</td>
<td>0.07</td>
</tr>
<tr>
<td>2.76 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>3</td>
<td>17.7</td>
<td>0.08</td>
</tr>
<tr>
<td>2.76 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>3</td>
<td>13.67</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Table 2
Detection of EHHV1 in clinical samples using real time SYBR Green I-based PCR.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Quantitative (copy/g of tissue or copy/ml of whole blood)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tongue</td>
<td>4.51 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lung</td>
<td>Negative</td>
</tr>
<tr>
<td>Heart</td>
<td>1.82 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kidney</td>
<td>Negative</td>
</tr>
<tr>
<td>Ovary</td>
<td>Negative</td>
</tr>
<tr>
<td>Lymph node</td>
<td>Negative</td>
</tr>
<tr>
<td>Spleen</td>
<td>4.54 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver</td>
<td>Negative</td>
</tr>
<tr>
<td>Whole blood</td>
<td>1.39 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
fragments were detected to confirm further the presence and quality of extracted DNA. All negative samples were β-actin positive with Ct values ranging between 21 and 38 (data not shown).

3.4. Sequencing and phylogenetic analysis

The positive trunk swab samples (EEHV11/54 and EEHV28/54) and the positive control were amplified by primers EmEEHV1 and EmEEHV2 for cloning and sequencing. The DNA sequences were compared with available sequences in GenBank. The results showed that the positive samples were 97–100% identical to EEHV1 terminase genes. The sequence homology of the positive control and the positive samples was 100%. To classify the subtype of EEHV1, a phylogenetic tree was constructed from 231 nucleotides of terminase gene sequences of EEHV. From the phylogram, EEHV isolated in this study were grouped into EEHV1A (Fig. 3).

4. Discussion

EEHV1 can cause hemorrhagic disease which attacks endothelial cells among Asian elephants causing a mortality rate of up to 85% (Latimer et al., 2011). The disease has become a very serious problem, especially in juvenile Asian elephants in zoos and camps (Ehlers et al., 2006; Reid et al., 2006; Richman et al., 1999, 2000). A rapid test with high sensitivity and specificity is useful to provide treatment and management of EEHV1 infection in captive Asian elephants. The SYBR Green I-based real-time PCR assay for EEHV1 detection was developed in this study. The test detected DNA as low as 276 copies/μl. CV values of intra- and inter-assay were less than 3%, indicating that the results are highly reproducible. This assay also showed that cross-reactivity did not occur with other mammalian herpesviruses. However, high specificity was demonstrated only with EEHV1. Other EEHV subtypes (EEHV2–6) were not investigated in this study due to a lack of positive samples in Thailand. The chances of cross-contamination between the positive control and the samples were minimized by following good laboratory practices. In the laboratory, the locations for DNA extraction, PCR setup, and DNA template adding were separated. Positive samples from real-time PCR assay were confirmed further by conventional PCR with other primer pairs (the primers used for sequencing) to amplify longer fragments. This evidence indicates that while performing the real time PCR, true
positive samples were not cross-contaminated. Two positive samples were sequenced and analyzed. The viruses were classified in the same group with other EEHV isolated in Thailand (FJ767711 and FJ515310) and grouped into EEHV1A. From the phylogenetic tree, EEHV1A is a major threat in Thailand. However, more sequences of EEHV isolated from Thailand should be determined to provide additional information.

From previously reports, EEHV1 was detected in trunk wash in healthy Asian elephants (Hardman et al., 2011; Stanton et al., 2010). However, there is a limitation to collecting trunk wash in healthy captive elephants. Sampling can only be performed in elephants that are trained. In this study, the virus was detected from trunk swab samples which provide an EEHV screening program for captive elephants to develop effective means of disease prevention.

In conclusion, the SYBR I-based real-time PCR assay has been shown to be rapid, sensitive, specific, and reproducible for detection and determination of quantity of EEHV1. It could be an excellent tool for laboratory detection, determination of epidemiology, and management and control of EEHV1.

References


