Short Communication

Zebra-borne equine herpesvirus type 1 (EHV-1) infection in non-African captive mammals

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1. Introduction

Equine herpesvirus type 1 (EHV-1), a member of the Varicellovirus genus in the subfamily Alphaherpesvirinae, is one of the most important pathogens of domestic horses worldwide (Lunn et al., 2009; Ma et al., 2013). Viruses closely related to EHV-1 have also been isolated from a number of equid species, including Persian onagers (Equus hemionus onager) (Ghanem et al., 2008), Damara zebra (Equus quagga), Grant’s zebra (Equus quagga boehmi), Burchell’s zebra (Equus quagga burchelli) and Grevy’s zebras (Equus grevyi) (Borchers et al., 2006; Ghanem et al., 2008; Ibrahim et al., 2007). A closely related but distinct virus, EHV-9, was first described in Thomson’s gazelles (Eudorcas thomsoni) suffering neurological symptoms (Fukushi et al., 1997), although it is suggested that equids are the natural and definitive host as many zebras were reported to be seropositive for EHV-9 (Schrenzel et al., 2008).

Herpesviruses are generally species-specific with limited reports of inter-species transmission. Both EHV-1 and EHV-9 have been shown to accomplish species jumps beyond their natural hosts in captivity resulting in infections of non-equid species (Chowdhury et al., 1988; Crandell et al., 1988; Rebhun et al., 1988; Schrenzel et al., 2008). Recently, a zebra-borne recombinant EHV-1 was detected in polar bears in Germany (Greenwood et al., 2012). In the case reported here a pregnant female Indian rhinoceros aborted in mid-pregnancy, suffered neurological disease and ultimately was euthanized. Viral DNA was detected in different tissues with nested and qPCR. Molecular characterization demonstrated high (99%) similarity with zebra-borne EHV-1 strain T-616 and Onager-derived EHV-1 (T-529) for the gB gene, and the
same level of identity to a previously reported EHV-1 isolated from a polar bear for five genes (Greenwood et al., 2012). The results are discussed in terms of the significance of trans-species transmission in zoological collections.

2. Materials and methods

2.1. Case history and sample collection

A 20-year-old female Indian rhinoceros (Purana) was housed with a male Indian rhinoceros (Ropen) and their daughter (Seto) in an indoor/outdoor enclosure at the Zoo Nuremberg, Germany. Purana had abortion in the mid-stage (after eight and half months) of pregnancy. Six days later, Purana suffered from rapid respiration and nervous manifestations. Samples, i.e. saliva from Seto and Ropen as well as brain, lung, spleen, and liver from Purana, were collected and stored at −80°C. A 2-year-old male polar bear (Gregor) in the same zoo showed loss of appetite, depression, heavy salivation, difficult breathing and nervous symptoms five weeks prior to Purana’s death. However, he recovered and subsequently exhibited good health and overall body condition. Saliva was collected from Gregor and two additional co-housed polar bears (Aleut and Vera).

2.2. PCR and cloning

PCR reactions were performed using MyTaq HS polymerase mix (Bioline), and approximately 115 ng of the extracted DNA as a template. Nested PCR amplification was performed using the same primer set (Supplementary Table 1) as described previously (VanDevanter et al., 1996). EHV-1-specific primers (Supplementary Table 1) that target various regions of several genes, including gB (UL27), IR6 (gene 67), UL45, UL49.5 and Pol (UL30), were also employed. The amplified products were purified and directly sequenced. Molecular cloning of the amplified fragments was performed using pGEM-T Vector System (Promega, USA). All DNA samples were analyzed by qPCR with the Applied Biosystems 7500 FAST (ABI, Foster City, CA) using specific primers and probes targeting the highly conserved gB gene (Supplementary Table 1) (Hussey et al., 2006).

2.3. Western blot analysis

Tissue lysates were prepared from different tissues and the proteins were separated using SDS-12% polyacrylamide gel electrophoresis (SDS-PAGE) (von Einem et al., 2007). The blots were incubated with rabbit polyclonal anti-IR6 antibody (1:10,000) (O’Callaghan et al., 1994) overnight at 4°C. Goat anti-rabbit IgG coupled to peroxidase (Southern Biotech, Birmingham) at 1:20,000 dilution was used as a secondary antibody. Reactive bands were visualized by enhanced chemiluminescence (Amersham ECL plus, GE healthcare).

2.4. Phylogenetic analysis

Phylogenetic analysis was based on nucleotide sequences of gB, IR6, UL45, UL49.5, and Pol (UL30) isolated from Purana’s tissues. Reference sequences for the same regions of EHV-1, EHV-9, and EHV-4 were obtained from GenBank and aligned using ClustalW implemented in Bioedit software (Thompson et al., 1994), the details of the aligned different strains are shown in supplementary Fig. 2A–E. Phylogenetic analysis of the alignments was performed using Maximum likelihood (ML) and Bayesian inference (BI) methods using EHV-4 as the outgroup. Bayesian information criterion (BIC) was used to determine the model of nucleotide substitution that best fit the data. The Hasegawa, Kishino and Yano (HKY) model was determined to be the best fit for the data base calculated by j-Model Test 0.1.1 (Posada, 2008). The Bayesian method implemented in the MrBayes 3.2.1 software (Anez et al., 2013; Huelsenbeck and Ronquist, 2001) was used to analyze the data set. The default number of Markov Chain Monte Carlo (MCMC) chains was run for 1,000,000 generations, sampling every 200 generations, generated majority consensus trees after a burn in of 1250 generations.

3. Results

3.1. Detection of EHV-1

A 250-bp fragment was detected in brain and lung tissues as expected for the nested PCR (supplementary Fig. 1A). Sequencing of the product revealed 97% identity at the nucleotide level to previously reported EHV-1 and EHV-9 Pol sequences. Specific amplified bands were observed for gB, Pol, UL45, UL49.5, and IR6 (supplementary Fig. 1B–F). Brain tissue was positive for all target genes. However, lung tissues were positive only for the gB and IR6 genes, the faint band with UL49.5 was non-specific. Amplification of viral DNA from spleen tissue gave faint bands with IR6- and UL49.5-specific primers (supplementary Fig. 1C and E). qPCR results for Viral DNA extracted from brain, lung, spleen, and liver tissues targeting gB are shown as threshold cycle (Ct) values (Table 1). We could detect gB-specific signals in DNA extracted from all tissues except the liver (Table 1), with brain demonstrating the lowest Ct values and, hence, the highest relative levels of EHV-1/ EHV-9 DNA. No qPCR signal was detected in saliva sampled from the co-housed rhinoceroses Seto and Ropen.

Saliva obtained from Gregor during the seizures and the recovery period were weakly positive with the EHV-1/ EHV-9-specific qPCR (Table 1). Gregor survived and affected tissues, such as brain and lung, could not be sampled. However, No qPCR signal was obtained using

<table>
<thead>
<tr>
<th>Name</th>
<th>Tissue sample</th>
<th>EHV-1 gB qPCR (Ct) values</th>
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<tbody>
<tr>
<td>Purana</td>
<td>Brain</td>
<td>21</td>
</tr>
<tr>
<td>Purana</td>
<td>Lung</td>
<td>31</td>
</tr>
<tr>
<td>Purana</td>
<td>Spleen</td>
<td>31</td>
</tr>
<tr>
<td>Purana</td>
<td>Liver</td>
<td>Negative</td>
</tr>
<tr>
<td>Gregor</td>
<td>Saliva</td>
<td>37</td>
</tr>
<tr>
<td>Aleut</td>
<td>Saliva</td>
<td>Negative</td>
</tr>
<tr>
<td>Vera</td>
<td>Saliva</td>
<td>Negative</td>
</tr>
<tr>
<td>H2O</td>
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<td>Negative</td>
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saliva sampled from the clinically co-housed unaffected polar bears.

3.2. Western blot analysis

IR6 protein was detected in Purana’s lung tissue and the included positive controls, and also matched the expected size of IR6 gene product of approximately 33-kD (Osterrieder et al., 1996). No IR6 protein was detected in the negative control rhinoceros lymph node tissue, other tissues obtained from Purana, or mock-infected ED cells (Fig. 1). However, the brain sample became gelatinous upon freezing and thawing, and protein retrieval was difficult. Although attempted, culturing of virus from the infected tissues failed. However, the tissues had been frozen and thawed repeatedly prior to the molecular analysis. This also prevented successful analysis by electron microscopy, immunohistochemistry and immunofluorescence, the latter giving extremely high non-specific background after multiple attempts (not shown).

3.3. Phylogenetic analysis

The pan-herpes PCR sequences were unable to definitively distinguish EHV-1 from EHV-9. In order to determine the identity of the virus present in Purana’s tissues and compare it with known EHV-1, EHV-9 and more distantly related EHV-4 sequences, The gB, IR6, UL45, UL49.5, and Pol PCR products were directly sequenced and from individual pGEM-T (Invitrogen)-derived clones (Chukeatirote et al., 2012). Clonal sequence analyses did not suggest co-infection with more than one strain. The amplified products were phylogenetically analyzed using Maximum Likelihood and Bayesian inference methods (Greenwood et al., 2012) (supplementary Fig. 2). At the nucleotide level, all sequences strongly supported that Purana’s sequences belong to an EHV-1 clade that includes the polar bear and zebra EHV-1 sequences and is more distant to horse EHV-1 strains or EHV-9 (Fig. 2). However, the sequence was not identical to the previously identified polar bear isolate for any sequences obtained, suggesting that zebra-derived EHV-1, while very similar to EHV-1 strains in general, may be diversifying in captive zebras. The nucleotide sequences obtained in this study were deposited in GenBank (accession numbers KF582612–KFS82616). Previous results (Greenwood et al., 2012) indicated that the Pol gene identified in the polar bear was a recombinant between EHV-1 and EHV-9, with the 5’ portion being EHV-1-like, the middle being EHV-9-like, and the last 110 bp again being EHV-1-like. The sequence in this region for Purana was nearly identical with this previously described sequence (2 bp differences at positions 213 and 813), indicating that the virus contains the same recombined polymerase region (supplementary Fig. 3).

4. Discussion

Zoos represent an artificial system with properties similar to habitat encroachment. Non-sympatric species are brought into close contact with each other. In theory, this environment could facilitate pathogen jumps and have catastrophic consequences if the affected populations belong to endangered species. This study demonstrates a cross-species transmission of zebra-borne EHV-1 infection into non-equid species.

Fig. 2. Phylogeny of EHV sequences detected in an Indian rhino. A summary tree for maximum Likelihood trees derived from gB (UL27), DNA polymerase (UL30), IR6, UL45 and UL49.5 is shown within a depiction of the EHV-1 genome and the relative position of the genes sequenced. Individual trees are shown in supplementary Fig. 2A–E.

Sequence of five EHV-1 genes from the rhinoceros Purana clearly demonstrates that she was infected by an EHV-1 strain most similar to one previously isolated from a polar bear from the Zoological Garden Wuppertal that caused illness in two polar bears in 2010 and fatality in one (Greenwood et al., 2012). The mode of transmission is unclear as the zebras and Indian rhinoceroses were not co-housed at the time of the infection. However, the distance between the rhinoceros and zebra enclosures is 15 m and transmission by aerosol may have been possible. It is worth noting that zebra and rhinoceros shared the same zoo keepers who could possibly be reflecting a source of transmission via fomites and clothing. Alternatively, it is possible that EHV-1 was transmitted by an unknown live vector, for example rodents that are ubiquitous in zoos.
While EHV-1 transmission between equids by fomites has been documented, a live vector was never implicated in EHV-1 spread. A screen of 25 trapped rodents for presence of the virus did not yield positive results (not shown). However, as mice (Mus musculus) are known to rapidly clear EHV-1 infection under experimental conditions (El-Nahass et al., 2012; Goodman et al., 2007), the negative result could reflect sampling of the animals at a time they were not shedding. We can conclude at present, however, that direct contact between zebras and rhinoceros was not required for transmission similar to other reported cases of EHV-1 infection in captive non-equids (Greenwood et al., 2012; Schrenzel et al., 2008).

A saliva sample obtained from Gregor was positive, albeit weakly, for EHV gB by qPCR. The two co-housed bears which did not exhibit any symptoms were negative. Since Gregor recovered, it was not possible to sample tissues to obtain more viral DNA sequences further complicated by the fact that saliva is a suboptimal source for EHV-1 isolation. While we were unable to determine whether Gregor was infected by EHV-1 or EHV-9 as the qPCR applied here is unable to distinguish between the two viruses, which we hypothesize that the two incidences were related given the relatively close association in time.

The present study is the first to describe the presence of EHV-1 DNA in a rhinoceros species. However, there is one previous report, which documented the prevalence of EHV-1/EHV-9-specific antibodies in free-ranging rhinoceros in Africa (Fischer-Tenhagen et al., 2000). As an important distinction to African rhinoceroses, Indian rhinoceroses would never come in contact with zebras in the wild. Thus, the mortality induced by EHV-1 in species that are non-con-specifics of zebras may suggest exposure to the virus may result in particularly severe outcomes for non-African mammals.

Indian rhinoceroses are listed by CITES as highly vulnerable, and, thus, threatened to go extinct (Fischer-Tenhagen et al., 2000). It is becoming clear that zoo populations are at risk of cross-species transmission of EHV-1, as different animal species from distinct geographic areas and habitats coexist within a very confined environment. It will be critical to establish regular molecular survey programs to investigate the spread of newly emerging viruses and to track the possible sources and ways of transmission of these viruses. Without this knowledge, EHV-1 and its close relative EHV-9 will likely continue to emerge with associated fatalities in collections of non-African mammals in particular and jeopardize efforts to conserve biodiversity in endangered species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jvetmic.2013.12.011.

References


