Aus der Abteilung für Wildtierkrankheiten
im Leibniz Institut für Zoo und Wildtierforschung
im Forschungsverbund Berlin e.V.

eingereicht beim Fachbereich Veterinärmedizin
der Freien Universität Berlin

Equine herpesvirus type 1 and 9 (EHV-1 and EHV-9) prevalence and biology in zoo and wild animals

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktor der Philosophie (PhD)
in den biomedizinischen Wissenschaften
im Dahlem Research School (DRS) Programm
an der Freien Universität Berlin

vorgelegt von
Azza Abdelgawad
Tierarztin aus Zagazig, Egypt

Berlin 2016

Journal-Nr.: 3921

Gedruckt mit Genehmigung des Fachbereichs Veterinärmedizin der Freien Universität Berlin

Dekan:	Univ.-Prof. Dr. Jürgen Zentek
Erster Gutachter:	Univ.-Prof. Dr. Alex Greenwood
Zweiter Gutachter:	Univ.-Prof. Dr. Klaus Osterrieder
Dritter Gutachter:	Univ.-Prof. Dr. Marcus Doherr

Deskriptoren (nach CAB-Thesaurus):
Equid herpesvirus 1, Equid herpesvirus 9, zebras, zoo animals, wild animals, seroprevalence, diversity, latent infections, recombination, disease transmission

Tag der Promotion: 12.09.2016

Coverbild: *zebra in the national park* © mhskypixel.jpg

In memory of my father

Dedicated to
my husband and my children
my mother and sisters

Supervisor:

Univ.-Prof. Dr. Alex Greenwood
Department of Wildlife Diseases, Leibniz Institute for Zoo and Wildlife Research, and Department of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany

Supervisor Committee:

Univ.-Prof. Dr. Alex Greenwood
Department of Wildlife Diseases, Leibniz Institute for Zoo and Wildlife Research, and Department of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany

Univ.-Prof. Dr. Klaus Osterrieder
Institut für Virologie, Robert von Ostertag-Haus, Zentrum für Infektionsmedizin, Freie Universität Berlin, Berlin, Germany

Dr. Marion L. East
Department of Evolutionary Ecology
Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany

This thesis is based on following manuscripts:

1. Azza Abdelgawad, Robert Hermes, Armando Damiani, Benjamin Lamglait, Gábor Á. Czirják, Marion East, Ortwin Aschenborn, Christian Wenker, Samy Kasem, Nikolaus Osterrieder, Alex D. Greenwood. Comprehensive Serology Based on a Peptide ELISA to Assess the Prevalence of Closely Related Equine Herpesviruses in Zoo and Wild Animals. PLos One. 2015 Sep 17;10(9):e0138370. doi: 10.1371/journal.pone.0138370. eCollection 2015- Chapter 2

2. Azza Abdelgawad, Armando Damiani, Simon Y.W. Ho, Günter Strauss, Claudia A. Szentiks, Marion L. East, Nikolaus Osterrieder, and Alex D. Greenwood. Zebra alphaherpesviruses: genetic diversity, latency and co-infections. *Viruses* (Under review) - Chapter 3

3. Azza Abdelgawad, Walid Azab, Armando Damiani, Katrin Baumgartner, Hermann Will, Nikolaus Osterrieder, Alex D. Greenwood. Zebra-borne equine herpesvirus type 1 (EHV-1) infection in non-African captive mammals. Vet Microbiol. 2014 Feb 21;169(1-2):102-6. doi: 10.1016/j.vetmic.2013.12.011. Epub 2013 Dec 25- Chapter 4

Contents

List of Figures

List of Abbreviations

Abbreviation	Full term
EHV-1	Equine herpesvirus type 1
EHV-9	Equine herpesvirus type 9
U_L	Unique long
U_S	Unique short
GHV-1	Gazelle herpesvirus-1
TR_S	Terminal repeats
IR_S	Internal repeats
ORF	Open reading frame
EHM	Equine herpesvirus myeloencephalopathy
N	Asparagine
D	Aspartic acid
Pol	DNA polymerase gene
SNT	Serum neutralization test
IFA	Indirect immunofluorescence assay
ELISA	Enzyme-linked Immunosorbent Assay
EHV-4	Equine herpesvirus type 4
gE	Glycoprotein E
gG	Glycoprotein G
gI	Glycoprotein I
gD	Glycoprotein D
gp2	Glycoprotein gp2
CNS	Central nervous system
HVS-1	Human herpesvirus type 1
HSV-2	Human herpesvirus type 2
ICP4	Infected cell protein 4
LATs	Latency-associated transcripts
ICP0	Infected cell polypeptide 0
mRNA	Messenger RNA
RT-qPCR	Quantitative real-time PCR
nRT–PCR	Nested- quantitative real-time PCR
SMLNs	Submandibular lymph nodes

Chapter 1

General introduction

1.1 Classification, history, and genome description

The order *Herpesvirales* includes three families: *Herpesviridae*, *Alloherpesviridae* and *Malacoherpesviridae* (Davison *et al.*, 2009). The family *Herpesviridae* consists of three subfamilies namely *Alpha*-, *Beta*- and *Gamma-herpesvirinae*, which infect mammals, birds and reptiles (Davison, 2007; Davison *et al.*, 2009).

In the *Equidae*, nine herpesviruses have been identified to date. Six equine herpesviruses (EHV-1, EHV-3, EHV-4, EHV-6, EHV-8, and EHV-9) belong to the subfamily *Alphaherpesvirinae* and three (EHV-2, EHV-5, and EHV-7) to the subfamily *Gammaherpesvirinae* (Davison *et al.*, 2009). EHV-1 and EHV-9 are double stranded DNA viruses that are classified within the subfamily *Alphaherpesvirinae*, genus *Varicellovirus* (Davison *et al.*, 2009; Fukushi *et al.*, 2012).

EHV-1 was isolated first from an aborted horse fetus in 1932. It was distinguishable from EHV-4, the highly genetic and antigeneticly related virus to EHV-1. EHV-1 can cause systemic disease ranged from rhinopneumonitis to abortion and myeloencephalopathy, while EHV-4 is limited to the upper respiratory tract (Dimock, 1933; Kydd *et al.*, 2006; Ma *et al.*, 2013; Reed & Toribio, 2004). EHV-9 was first described and isolated in an outbreak in Japan in Thomson's gazelles (*Gazelle thomsoni*) (Fukushi *et al.*, 1997). The virus was first designated as gazelle herpesvirus-1 (GHV-1). Nucleotide sequence analyses revealed that EHV-9 had high genetic similarity to EHV-1; however, DNA fingerprints were distinct from those of EHV-1 and other equine herpesviruses.

The whole genomes of both EHV-1 and EHV-9 had been sequenced (Fukushi *et al.*, 2012; Kasem *et al.*, 2008; Telford *et al.*, 1992). The genomes of EHV-1 and EHV-9 are approximately 150 kbp and 148,371 bp in size, respectively. The genomes have unique long (U_L) and unique short (U_S) sequences linked to each other and flanked by inverted terminal and internal repeat regions (TR_S/IR_S) (Telford *et al.*, 1992; Telford *et al.*, 1998). EHV-1 and EHV-9 have 76 genes with 80 open reading frames (*ORFs*) due to the duplication of four genes (namely, *ORF-64*, *-65*, *-66*, and *-67*) that flank the U_S region. As mentioned above, there is high genetic similarity between EHV-1 and EHV-9; the overall percent identity between their complete genome sequences is 93%. *ORFs 42*, *52*, and *53* revealed the highest degree of identity (95%), while *ORF71* encoding glycoprotein *gp2* revealed the lowest degree of identity (86%) (Fukushi *et al.*, 2012).

Several studies investigated the virulence of EHV-1 strains determining the ability of the virus to induce nervous manifestations, equine herpesvirus myeloencephalopathy (EHM), and leading to the classification of EHV-1 into neurovirulent and non-neurovirulent strains. It was reported that the ability of the neurovirulent strains to infect endothelial cells, causing subsequent complications, are related to the higher replication efficiency of these viruses compared to the non-neurovirulent strains (Damiani *et al.*, 2014). EHM was observed to be associated with a single substitution changing asparagine (N) to aspartic acid (D), at amino acid position 752 (D752) of *ORF30* encoding the viral DNA polymerase gene (*Pol*). On the nucleotide level, a single nucleotide polymorphism A/G2254 in the same gene was detected (Goodman *et al.*, 2007). However, not all cases infected with EHV-1 with the point mutation D752 suffered from neurological symptoms (Crabb *et al.*, 1995).

1.2 Epidemiology

1.2.1 Host range in zoo and wild animals

EHV-1, arguably one of the most important equine pathogens, is distributed throughout the world among horse populations. Although EHV-1 infections are usually limited to equine species, the virus can also infect different species of non-equids, particularly, in zoos (Chowdhury *et al.*, 1986; Chowdhury *et al.*, 1988; Crandell *et al.*, 1988; Kinyili & Thorsen, 1979; Rebhun *et al.*, 1988; Wohlsein *et al.*, 2011; Wolff *et al.*, 1986).

The relationship between EHV-1 and its equine host is complex and not fully understood. Different virus strains with low level of genetic variability have been identified (Gardiner *et al.*, 2012; Tearle *et al.*, 2003; van Maanen *et al.*, 2001). Apart from domestic horses (*Equus ferus caballus*), variant EHV-1 has also been isolated from different equine species, including captive and wild equids. Previous reports have shown that viruses closely related to EHV-1 were isolated from a fetus of Persian onager *(Equus hemionus onager)* (strain T529), which was aborted after 10 months of gestation in the National Zoological Park in Washington (Ghanem *et al.*, 2008). EHV-1 isolates from a 5-year-old female Grevy's zebra *(Equus grevyi)* in the Park Zoo in Chicago and a Grevy's zebra stallion in England were detected (strains were designated T965 and T616 for the two Grevy's zebra isolates, respectively). EHV-1 infection was also identified in Plains zebra, Damara zebra *(Equus quagga antiquorum)*, Grant's zebra *(Equus quagga boehmi)* and Burchell's zebra *(Equus quagga burchelii)* (Borchers *et al.*, 2006; Ghanem *et al.*, 2008; Ibrahim *et al.*, 2007; Wolff *et al.*, 1986). Moreover, antibodies for EHV-1 were detected in other distantly related perissodactyls including African white (*Ceratotherium simum*) and black (*Diceros bicornis*)

rhinoceroses and in an Indian tapir (*Tapirus indicus*) indicating exposure to virus infection (Fischer-Tenhagen *et al.*, 2000; R.Göltenboth *et al.*, 1996).

Previous studies have shown that infection of non-natural hosts with neurotropic alphaherpesviruses can cause fatal infections. Cross species jumps are often characterized by enhanced neurovirulence as was shown in the case of human herpes simplex virus in rabbits (Weissenbock *et al.*, 1997) or chinchillas (Wohlsein *et al.*, 2002), herpes B virus (cercopithecine herpesvirus 1) in humans (Elmore & Eberle, 2008) or pseudorabies virus in cats, dogs and bovines (Salwa, 2004). EHV-1 has also been isolated from non-equid species including captive black buck *(Antilopa cervicapra)*, cattle *(Bostaurus)*, deer *(Dama dama)*, alpacas (*Vicugn apacos*), and llamas (*Lama glama*) in New York state (Borchers *et al.*, 2006; Chowdhury *et al.*, 1988; Kinyili & Thorsen, 1979). A recent study has identified neurotropic EHV-1 in Thomson's gazelles, black bear *(Ursusamericanus)*, and guinea pigs *(Caviaporcellus f. dom.)* in two different zoo epizootics and were associated with abortion, severe neurological symptoms and high mortality rates (Wohlsein *et al.*, 2011).

EHV-9 is another closely related but yet distinct virus and was first detected in an outbreak in Japan in seven Thomson's gazelle. One 4-year-old female was found dead without showing any clinical symptoms, followed by a series of neurological signs in another 6 gazelles. However, gazelle was considered an accidental host (Fukushi *et al.*, 1997). Furthermore, EHV-9 was also detected in two Grevy's zebras with respiratory lesions in the zoological garden in San Diego, California. When these two zebras were moved in an enclosure near to a polar bear cage, the polar bear died later on due to EHV-9 infection (Schrenzel *et al.*, 2008). EHV-9, like EHV-1, can cause disease in a variety of other mammals either naturally or experimentally (Borchers *et al.*, 2008; Fukushi *et al.*, 1997; Kasem *et al.*, 2008; Kodama *et al.*, 2007; Yanai *et al.*, 2003a; Yanai *et al.*, 2003b). EHV-9 was detected in a reticulated giraffe *(Giraffa camelopardalis reticulate)* which suffered from neurologic symptoms before death (Kasem *et al.*, 2008). To date, neither the host range nor the natural host of EHV-9 is known. The virulence of EHV-9 infection and the potential jumps between different animal species raise the importance of experimental studies which showed the ability of the virus to induce disease in members of different Mammalia including, dogs, cats, mice, hamster, pigs, goats, and horses (Taniguchi *et al.*, 2000a; Taniguchi *et al.*, 2000b; Yanai *et al.*, 2003a). Recently, a recombinant zebra-EHV-1/EHV-9 infection was reported in two polar bears (*Ursus maritimus)*, which had epileptic seizures with fatal outcome for the 20-year-old female, Jerka. The other bear, 16-year-old male, Lars, recovered after medical intervention (Greenwood *et al.*, 2012). The complete host range of

EHV-1, EHV-9, and the new EHV-1-recombinant strain and whether there are differences in captivity that potentially promote cross-species transmission remains unknown.

1.2.2 Seroprevalence of EHV-1 and EHV-9 in zoo and wild animals

EHV-1 and EHV-9 are the causative agents for rhinopneumonitis and menengioencephalomylitis. The difference between EHV-1 and EHV-9 has been demonstrated by sequencing of the whole viral genome (Fukushi *et al.*, 2012). However, because of high antigenic cross reactivity it is difficult to distinguish between the two viruses using conventional serological tests (Borchers *et al.*, 2005). The sero-epidemiological studies on EHV-1and EHV-9 infections in free-living animal populations other than horses, including zebra and rhinoceros species, are relatively few in number (Borchers & Frolich, 1997; Borchers *et al.*, 2005; Fischer-Tenhagen *et al.*, 2000).

The seroprevalence of EHV-1 and EHV-9 in zoo and wildlife is very limited and based on serum neutralization (SNT) and indirect immunofluorescence (IFA) assays. In Namibia, EHV-1 antibodies were detected in free ranging mountain zebras. EHV-1 positive antibodies were detected in all tested animals (n=21) using IFA, while neutralizing antibodies, using SNT, were detected in only three (14%) of the tested animals. Neither clinical signs nor viral DNA were detected in the tested animals (Borchers & Frolich, 1997). In Tanzania, serological studies indicated that wild Burchell's zebras from Serengeti National Park developed neutralizing antibodies to EHV-1 and EHV-9 infections at prevalence of 14% and 60%, respectively, without evidence of associated mortality (Borchers *et al.*, 2008). The high prevalence of EHV-9 in free ranging Burchell's zebra based on SNT suggests that Burchell's zebra may be a natural host for EHV-9. However, in all cases genetic characterization of viruses belonging to the detected neutralizing antibodies was not available. Furthermore, EHV-1 antibodies were detected in African white and black rhinoceroses at a prevalence of 8.8% based on the IFA (Fischer-Tenhagen *et al.*, 2000). In captivity, little is known about the sero-epidemiology of either virus. A few studies have detected EHV-1 and EHV-9 antibodies in random animal species. Out of 23 alpacas and llamas, antibodies for EHV-1 were detected in 21 animal sera. EHV-1 neutralizing antibodies were detected in onagers, mountain zebras, wild asses, impala and eland (Wohlsein *et al.*, 2011).

EHV-1 and EHV-9 are very similar genetically and antigenically; thus, SNT's have poor discriminatory power for such closely related viruses and results should be cautiously interpreted. Recently, a peptide-ELISA assay has been successfully applied to accurately

distinguish between the seroprevalence of highly similar virus strains such as EHV-1 and EHV-4 (Crabb *et al.*, 1995; Lang *et al.*, 2013; Yasunaga *et al.*, 1998). A type-specific-gG-based enzyme-linked immunosorbent assay (ELISA) was developed to differentiate between EHV-1 and EHV-4 antibodies (Crabb *et al.*, 1992; Crabb & Studdert, 1993). Following the same strategy, other ELISAs were developed to discriminate between EHV-1 and EHV-4 using specific peptides [glycoprotein E (*gE*) for EHV-1 and glycoprotein G (*gG*) for EHV-4] (Lang *et al.*, 2013; Yasunaga *et al.*, 1998). Thus, a specific peptide-based ELISA can, theoretically, make the study of EHV-1 and EHV-9 sero-epidemiology feasible.

1.2.3 Molecular epidemiology of EHV-1 and EHV-9 in zoo and wild animals

EHV-1 has been recognized for over 60 years as an ubiquitous pathogen in horse populations worldwide and is considered a threat to the international horse industry. Several studies have been undertaken to investigate, molecularly, the occurrence of EHV-1. Variant EHV-1 has been isolated from different equine species. In the United States, EHV-1-related viruses were isolated from aborted fetuses of Persian onager, Grevy's zebra and a Thomson's gazelle, which were kept near plains zebras. Neurological illness and non-suppurative encephalitis associated with infection was recorded in all cases. Investigations using nucleotide sequences of the *gG* gene indicated that the isolates belong to a genetic group that is distinct from EHV-1 isolated from horses (Ibrahim *et al.*, 2007). In another study, restriction enzymes and phylogenetic analyses based on different glycoproteins, *gB*, *gE*, and *gI*, and the tegument proteins *ORF8*, *ORF15* and *ORF68*, suggested that the gazelle isolate was similar to onager and zebra isolates, which was identical to an EHV-1 isolate from a blackbuck that died from signs of malignant catarrhal fever and showed a significant DNA homology with EHV-1 (Borchers *et al.*, 2006; Ghanem *et al.*, 2008). Molecular and epidemiological evidence suggested that EHV-1 was transmitted from zebras to gazelles. DNA restriction profile and blot hybridization analysis were used to characterize two ruminant isolates as EHV-1 (Chowdhury *et al.*, 1988). A recent study has identified neurotropic EHV-1 in four black bears, two Thomson's gazelles and 18 guinea pigs which died of neurological disease in two different zoo epizootics. Nucleotide sequences and phylogenetic analysis of the tegument protein (*ORF15*) revealed that the bear isolate clustered with EHV-1 isolates from horses, while the Thomson's gazelle and guinea pig isolates clustered with EHV-1 isolates from onager and zebra in a separate group (Wohlsein *et al.*, 2011). Molecular characterization of the EHV-9 genome, which was sequenced from gazelle, revealed high genetic similarity with EHV-1 (Fukushi *et al.*, 2012). Excluding

experimental studies, EHV-9 infection was not detected before in horses (Taniguchi *et al.*, 2000a). However, EHV-9 was detected in two Grevy's zebras before a polar bear developed progressive neurologic signs (Schrenzel *et al.*, 2008)**.** Sequencing of 742 bp and 1,181 bp of the *pol* and *gB* genes, respectively, together with phylogenetic analyses showed that the sequence from both Grevy's zebras and polar bear was EHV-9 (Fig. 1.1) (Schrenzel *et al.*, 2008). Furthermore, partial amplification of four different genes, *pol*, *gB*, *gD*, and *gp2*, revealed that the giraffe isolate belongs to EHV-9 (Kasem *et al.*, 2008). Recently, the full genome of EHV-1 isolated from zebra, onager, and gazelle has been sequenced (Guo *et al.*, 2014). Several studies referred to the zebra as the natural host of EHV-9. However, less genetic information is available for EHV-9 than that of EHV-1 (Borchers *et al.*, 2008; Rebelo *et al.*, 2015; Schrenzel *et al.*, 2008). The large population size of zebras together with the wide host range of EHV-1 and EHV-9 may increase genetic diversification and evolution of both viruses which may have pathogenic consequences in zebras. Studying genetic characterization of the circulating isolates and whether there is a difference between zoo and wildlife is crucial in terms of epidemiology and management measurements.

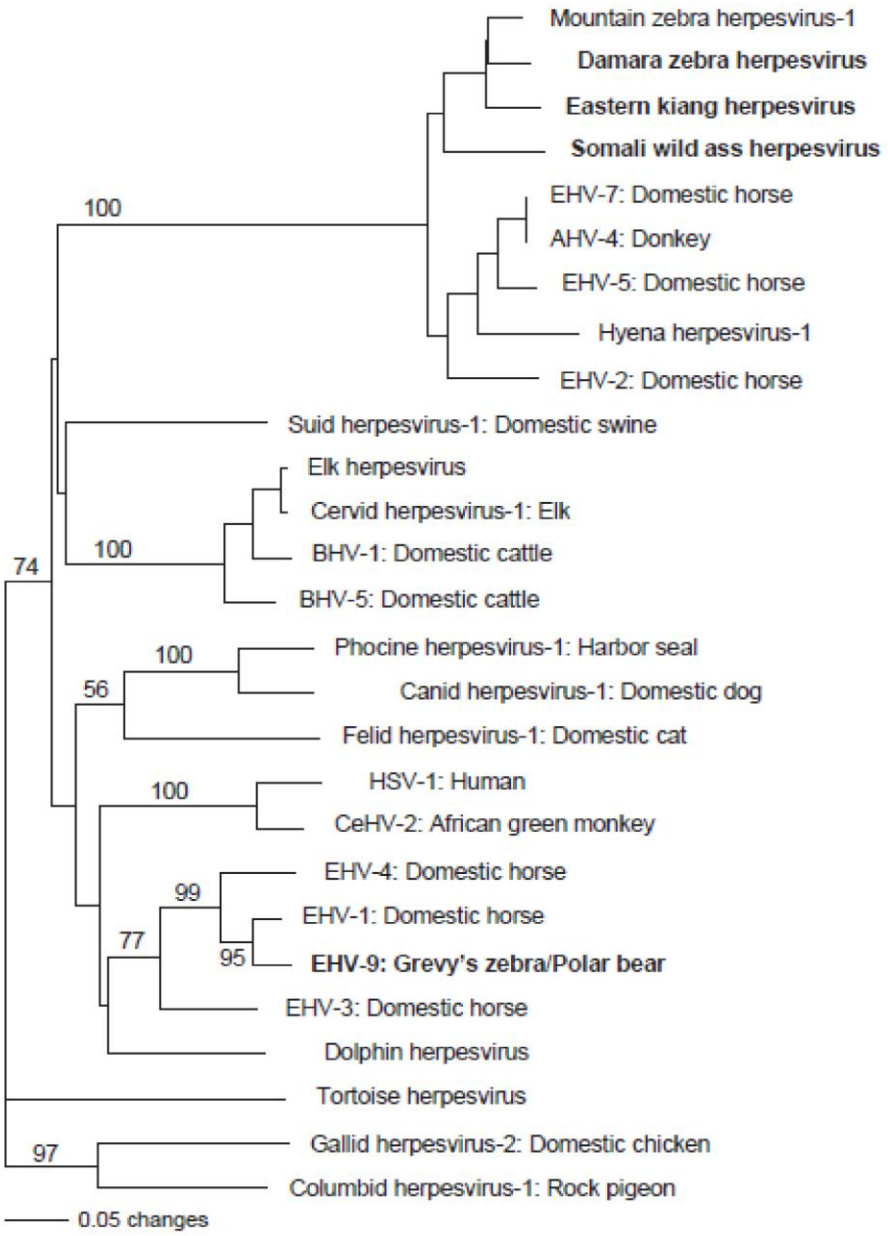


Fig. 1.1 Phylogeny of EHV-9 isolated from Polar bear. A phylogram of all equine herpesviruses and related viruses from other animals and their respective hosts created from a predicted amino acid segment of the DNA polymerase gene is shown. All sequences obtained from polar bear and Grevy's zebra are in boldface; bootstrap values >1,000 replicates are indicated; adapted from (Schrenzel *et al.*, 2008) with permission from Emerging Infectious Diseases.

1.3 Virus transmission

EHV-1 can be transmitted by inhalation, direct contact, fomites, and virus aerosols after shedding of the infectious virus with respiratory secretions. It has been shown that the aborted fetuses and the placenta after EHV-1 induced abortion could play an important role in virus transmission (Dunowska, 2014b; Lunn *et al.*, 2009; Patel *et al.*, 1982). EHV-1 DNA was detected in 13% of tested semen samples using PCR (Hebia-Fellah *et al.*, 2009). In another study, EHV-1 shedding was detected in semen of naturally infected stallions. However, infectious virus was not isolated in either study. Therefore, the role of semen in virus transmission and whether the vertical transmission of the virus is possible is unclear (Ma *et al.*, 2013; Walter *et al.*, 2012). Several studies have suggested zebras are the potential source of infection for both EHV-1 and EHV-9. However, transmission routes in zoos and wildlife are unknown, particularly in the absence of direct contact with other equids including zebras. Virus transmission through a live vector such as zookeepers and circulating rodents in zoos cannot be excluded. Shared water pools where the animals congregate could be a source of virus transmission. Detection of the source and mode of virus transmission is important for both viruses in terms of control and hygienic measures.

1.4 Pathology and pathogenesis

EHV-1 infection occurs regularly with respiratory disease, abortion, neonatal death and neurological disorders (Reed & Toribio, 2004). The primary site of infection is respiratory epithelium and the infection usually results in fever, depression, anorexia and nasal discharge that often ranged from serous to mucopurulent (Damiani *et al.*, 2014; Dunowska, 2014a; Gibson *et al.*, 1992). The virus is carried by infected mononuclear cells to the endothelial cells of the uterus and central nervous system (CNS) blood vessels causing tissue damage, thrombosis and/or hypoxia resulting in abortion or neurological disease (Allen & Bryans, 1986; Damiani *et al.*, 2014). Intra nuclear inclusion bodies in respiratory epithelium, degenerative vasculitis of small blood vessels in the brain or spinal cord with infiltration by inflammatory cells (mainly lymphocytes), and degenerative neurons are the main histopathological features of EHV-1 (Stierstorfer *et al.*, 2002).

Several studies showed the ability of EHV-1 to induce lesions in species other than equids. Non suppurative meningoencephalitis with intranuclear inclusion bodies in a degenerated neurons infected with EHV-1 were detected in alpacas which had signs of blindness and neurologic dysfunction, and in black bears reported in another study (Rebhun *et al.*, 1988; Wohlsein *et al.*, 2011).

Several studies, using different experimental animals, have been conducted to identify the pathogenesis of EHV-9. In experimentally infected horses, the common features between EHV-9 and EHV-1 infections of the nervous system were cellular reactions including perivascular cuffing and glial reactions. However, the neuropathology of horses inoculated with EHV-9 lacked thrombosis and vasculitis which are the most prominent histopathological features of EHV-1-associated with meningoencephalomyelitis (Allen & Bryans, 1986; Jackson *et al.*, 1977; Taniguchi *et al.*, 2000a). In general, experimental infection with EHV-9 has shown tropism for neural and respiratory tissues and is reflected by non-suppurative encephalitis with neuronal necrosis, intranuclear inclusion bodies, and gliosis in a wide spectrum of animals (Fukushi *et al.*, 2000; Fukushi *et al.*, 1997; Kodama *et al.*, 2007; Taniguchi *et al.*, 2000b; Yanai *et al.*, 2003a; Yanai *et al.*, 2003b). EHV-9 may traffic to the CNS either through the olfactory and trigeminal nerves or through alternative routes, for example by the systemic (vascular) route (El-Habashi *et al.*, 2010; El-Habashi *et al.*, 2011; El-Nahass *et al.*, 2011; Taniguchi *et al.*, 2000a). Taken together, our understanding of EHV-9 pathogenesis is incomplete and one can argue that neither the primary site of EHV-9 propagation nor the pathway the virus takes to arrive at the brain is known. Viral interstitial pneumonia with rhinitis and intranuclear inclusion bodies were detected in two Grevy's zebras infected with EHV-9 (Fig. 1.2) (Schrenzel *et al.*, 2008). Pathological lesions in polar bear, Thomson's gazelles, and giraffes naturally infected with EHV-9 (Fukushi *et al.*, 1997; Kasem *et al.*, 2008) ranged from moderate to severe non-suppurative encephalitis. The cerebrum was the main region where the histopathological changes, neuronal degeneration, perivascular cuffing, glial reactions, and intranuclear inclusions were

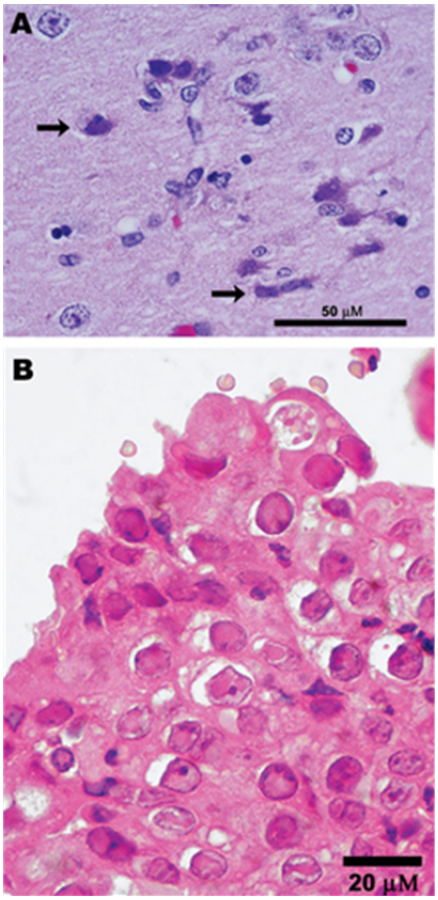


Fig. 1.2 Histopathology of EHV-9. (A) encephalitis with neuronal necrosis and intranuclear inclusions (arrows) in a polar bear; (B) Grevy's zebra with acute rhinitis with eosinophilic inclusions in respiratory epithelium; HE; adapted from (Schrenzel *et al.*, 2008) with permission from Emerging Infectious Diseases.

observed. Immunohistochemistry revealed a positive reaction in the neurons of the affected gazelles using EHV-1 immunolabelling (Fukushi *et al.*, 1997).

1.5 Natural recombination

Recombination is a process by which exchange of genetic materials result in unique genetic combinations of two chromosomes or genomes. Mixing genetic material (recombination or reassortment) is a widely adopted mechanism by RNA viruses to swap genes or polymorphisms among genomes. This is very clear with influenza viruses and some other RNA viruses such as retroviruses (Stamatakis, 2014). The recombination rate of DNA viruses is lower than that in RNA viruses. In general, DNA viruses have larger genomes than RNA viruses with high fidelity of the enzymes involved in the replication process (Escalera-Zamudio *et al.*, 2015; Tamura *et al.*, 2007). Numerous mammalian and avian viruses within the subfamily *Alphaherpesvirinae* show low rates of nucleotide substitution (Thiry *et al.*, 2005). Recombination events in alphaherpesviruses with high genetic similarity are an effective way for them to increase their genetic diversity given their low mutation rates.

Experimental data have shown that the recombination of alphaherpesviruses occurs frequently resulting in highly virulent recombinant strains (Javier *et al.*, 1986). Human herpesviruses (HVS-1 and HSV-2), which share 75% similarity show higher rates of recombination than members of the same subfamily with lower similarity under experimental conditions (Allen & Yeargan, 1987). Viruses co-infection is required for recombination to take place (Stamatakis, 2014). Several factors can affect the recombination pattern including biological, genetic, and epidemiological factors. The dose of inoculated virus, time interval between the two virus infections, genetic homology, host range, prevalence of the virus infection, virulence and latent infection interrupted by reactivation are important factors that should be considered in recombination (Thiry *et al.*, 2005). High prevalence of alphaherpesviruses infection increases the chance of recombination. In general, the mechanism of virus recombination is poorly understood in herpesviruses, particularly, viral and cellular factors that might be required (Dutch *et al.*, 1995; Taylor & Knipe, 2004). Evidence of recombination has been reported for EHVs. Natural recombination between EHV-1 and EHV-4 in the infected cell protein 4 (*ICP4)* gene was detected (Pagamjav *et al.*, 2005). The recombination between EHV-1 and EHV-9 viruses was also reported. A recombination break point was detected in the DNA polymerase gene with the 5' portion being EHV-1-like (80 bp), the middle being EHV-9-like (618 bp), and the end (110 bp) again being EHV-1-like. The new recombinant EHV-1 zebra strain may have been responsible for a

polar bear fatality in 2010 (Greenwood *et al.*, 2012). Whether this recombination occurred in free living or zoo zebras is unclear.

1.6 Latency

Members of the family *Alphaherpesvirinae*, including EHV-1, have the ability to establish a lifelong latent state in neurons of the sensory ganglia or in lymphoid cells (Aleman *et al.*, 2012; Pusterla *et al.*, 2009; Smith *et al.*, 1998). Trigeminal neuronal latency is likely to be established by transport of the virus through sensory nerve endings in the nasal cavity. The normal expression of viral genes in the lytic state is repressed in latency that gives herpesviruses the advantage of avoiding elimination by the host immune system (Allen, 2004; Ma *et al.*, 2013; Ramakrishnan *et al.*, 1996; van der Meulen *et al.*, 2006). A single region has been found to be transcriptionally active during latency, producing non-polyadenylated latency-associated transcripts (LATs) (Ramakrishnan *et al.*, 1996). LATs expression has become a hallmark of latent infections in herpesviruses. LATs have been shown to be transcribed from the DNA strand opposite to that encoding the infected cell polypeptide 0 (*ICP0*) and *ICP4* messenger RNAs (mRNA) (Baxi *et al.*, 1995; Pusterla *et al.*, 2009; Ramakrishnan *et al.*, 1996). The ability of EHV-1 to establish infection in the early stage of a foals' life followed by latent infection of recovered animals is an important epidemiological advantage for the virus (Dunowska, 2014b; Welch *et al.*, 1992).

Quantitative real-time PCR (RT-qPCR) analysis targeting a late gene, such as the envelope *gB* gene with absence of specific transcripts present during replication, may distinguish between viral replication and latency. Detection of active transcription of LATs at the RNA level, allow the confirmation of a latent state (Allen *et al.*, 2008; Pusterla *et al.*, 2010a; Pusterla *et al.*, 2012; Pusterla *et al.*, 2009). The latent infection of EHV-1 and EHV-4 has been reported in horses in several studies (Allen, 2006; Allen *et al.*, 2008; Borchers *et al.*, 1997; Pusterla *et al.*, 2012; Pusterla *et al.*, 2010b; Pusterla *et al.*, 2009). Although the site of latency was considered to be in lymphoid tissue (Carvalho *et al.*, 2000; Smith *et al.*, 1998), some studies have shown the ability of EHV-1 and EHV-4 to establish latent infection in the trigeminal ganglion (Borchers *et al.*, 1997; Borchers *et al.*, 1999; Ma *et al.*, 2013; Slater *et al.*, 1994). Specific EHV-4- transcripts were detected in 26% of tested trigeminal ganglion of horses using RT–PCR (Borchers *et al.*, 1999). EHV-1 has shown the ability to establish latent infection in different sites including blood, submandibular lymph nodes (SMLNs), bronchial lymph node, and trigeminal ganglion (Allen, 2006; Allen *et al.*, 2008; Pusterla *et al.*, 2012;

Pusterla *et al.*, 2010b). Indirect evidence of EHV-9 latency in the trigeminal ganglion of free living Burchell's zebras was also reported (Borchers *et al.*, 2008).

The periodic reactivation of latent infections, particularly under stressful conditions, leads to viral shedding and a chain of horizontal transmission (Edington *et al.*, 1985; Pusterla *et al.*, 2009). Sporadic cases of abortion and neurological disease can occur without exogenous infection supported by the observation that the majority of EHV-1 abortions occur as single events (Crowhurst *et al.*, 1981; Slater, 2007). Therefore, early recognition of infection is a crucial issue in terms of management to decrease the possible risk of virus exposure of susceptible animals.

1.7 Aim of the study

The aim of this study is to investigate the prevalence and the genetic diversity (recombination or natural mutation) of EHV-1 and EHV-9 circulating in zoo and wildlife, particularly in zebras. Although zoos provide a good health care and a high quality of life to animals, zoo animals are still at risk because of the newly emerging viral diseases, including EHV-1. The main hypothesis is: equid-inhabitants of zoos, particularly, zebras can serve as a host for both EHV-1 and EHV-9. After lytic replication ceases, latency occurs either in sensory trigeminal ganglion or lymphoid cells. The viruses can be reactivated under stress conditions causing re-infection and shedding. Since infection of natural hosts with more than one herpesvirus is the rule rather than the exception, zebras can provide a suitable environment for recombination of different EHV to take place, resulting in new strain(s) that may have novel or expanded host ranges (Fig. 1.3).

To address this hypothesis, I had three main goals:

1. EHV epidemiological survey

I conducted an epidemiological survey among zebra populations, non-equid zoo animals and wild African mammals (Fig. 1.4) to detect the prevalence of EHV-1 and EHV-9. For that purpose, a discriminatory peptide-based ELISA test was applied for 428 sera obtained from different animal species. I characterized the wide seroprevalence of EHV-1 and EHV-9 to include members of *Equidae*, *Rhinocerotidae* and *Bovidae* as described in chapter 2.

2. Diversity and latency among EHV-1 and EHV-9 strains in zebras

A genetic analysis of the circulating EHV-1 and EHV-9 isolates among zoo and wild zebras was done through sequencing of different genes to assess diversity rates among

viruses. Since EHV-1 and EHV-9 can infect zebras, I focused on the ability of the viruses to co-infect zebra and the ability of both viruses to establish latency in the sensory neuronal ganglia as demonstrated in chapter 3.

3. Natural mutation and/or recombination between EHV-1 and EHV-9 in infected animals

As a result of virus diversity and possible co-occurrence of both viruses in zebra, recombination can happen resulting in new virus strain(s) which can infect unexpected hosts causing fatality. Screening of a possible existence of a recombinant virus circulating among zoo and wild animals was carried out during the whole study. Among the screened cases, one new case was shown to be infected with a recombinant EHV-1/EHV-9 strain. This case report was demonstrated in detail in chapter 4.

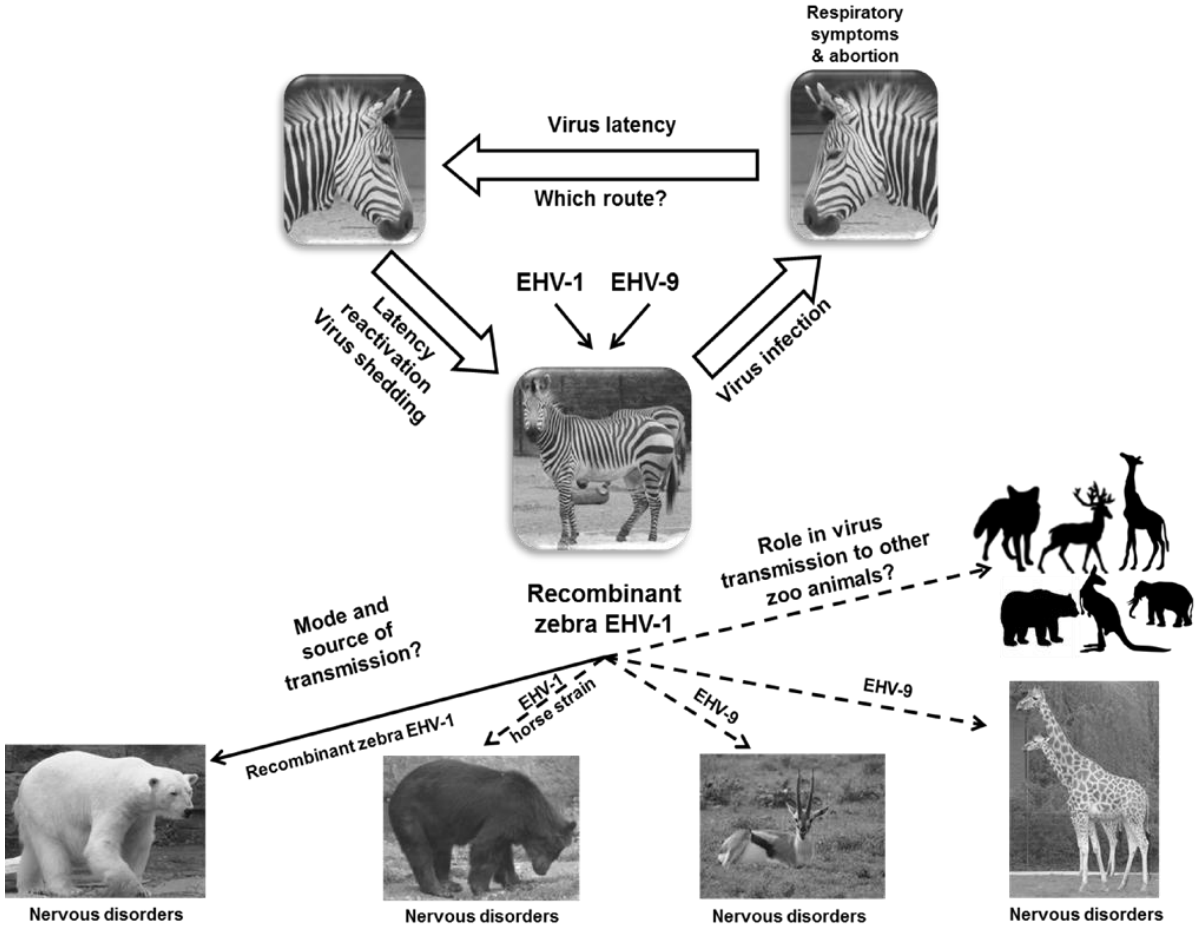


Fig. 1.3 Illustrate diagram for work hypothesis. Model for work hypothesis illustrates how zebra can be a host and source of infection for EHV-1 and EHV-9. Zebra can be a host where the recombination of both viruses can happen and infect animals such as polar bears (solid line) or unexpected animals (dash line); clipart black pictures are freely available from http://www.iconsdb.com; gazelle picture is courtesy of Peter Seeber; other pictures are taken by the author.

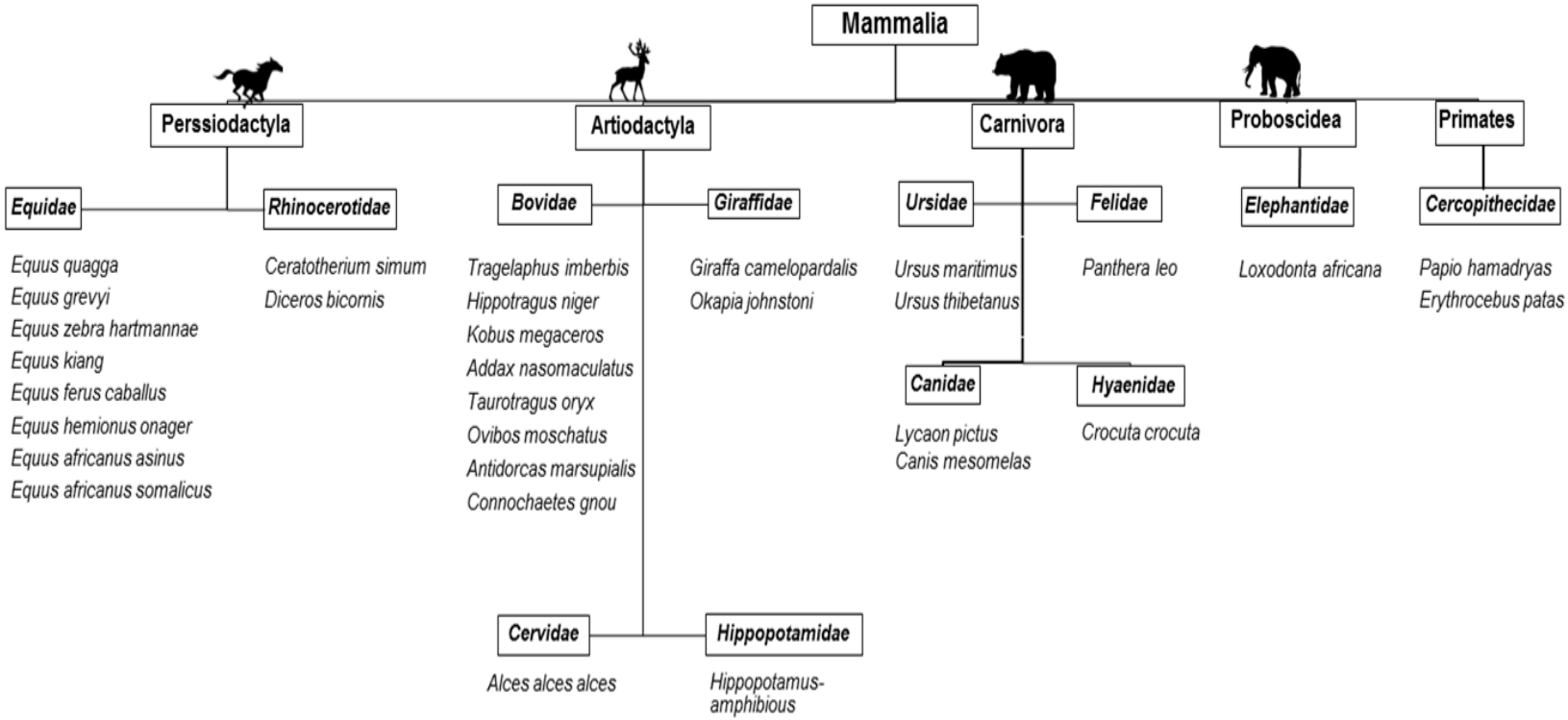


Fig 1.4 Species included in the study, clipart black pictures are freely available from http://www.iconsdb.com

1.8 References

Aleman, M., Pickles, K.J., Simonek, G. and Madigan, J.E. 2012. Latent equine herpesvirus-1 in trigeminal ganglia and equine idiopathic headshaking. *Journal of veterinary internal medicine / American College of Veterinary Internal Medicine,* **26**: 192-194.

Allen, G.P. 2004. *Equid herpesvirus- 1 (EHV-1) and -4 (EHV-4) infections. IN Infectious Diseases of Livestock, (Ed.) JAW Coetzer and RC Tustin. Oxford Press (Cape Town), Chapter 76, pp 829-859.*

Allen, G.P. 2006. Antemortem detection of latent infection with neuropathogenic strains of equine herpesvirus-1 in horses. *American journal of veterinary research,* **67**: 1401-1405.

Allen, G.P., Bolin, D.C., Bryant, U., Carter, C.N., Giles, R.C., Harrison, L.R., Hong, C.B., Jackson, C.B., Poonacha, K., Wharton, R. and Williams, N.M. 2008. Prevalence of latent, neuropathogenic equine herpesvirus-1 in the Thoroughbred broodmare population of central Kentucky. *Equine veterinary journal,* **40**: 105-110.

Allen, G.P. and Bryans, J.T. 1986. Molecular epizootiology, pathogenesis, and prophylaxis of equine herpesvirus-1 infections. *Prog Vet Microbiol Immunol,* **2**: 78-144.

Allen, G.P. and Yeargan, M.R. 1987. Use of lambda gt11 and monoclonal antibodies to map the genes for the six major glycoproteins of equine herpesvirus 1. *J Virol,* **61**: 2454-2461.

Baxi, M.K., Efstathiou, S., Lawrence, G., Whalley, J.M., Slater, J.D. and Field, H.J. 1995. The detection of latency-associated transcripts of equine herpesvirus 1 in ganglionic neurons. *The Journal of general virology,* **76 (Pt 12)**: 3113-3118.

Borchers, K., Bottner, D., Lieckfeldt, D., Ludwig, A., Frolich, K., Klingeborn, B., Widen, F., Allen, G. and Ludwig, H. 2006. Characterization of equid herpesvirus 1 (EHV-1) related viruses from captive Grevy's zebra and blackbuck. *The Journal of veterinary medical science / the Japanese Society of Veterinary Science,* **68**: 757-760.

Borchers, K. and Frolich, K. 1997. Antibodies against equine herpesviruses in free-ranging mountain zebras from Namibia. *Journal of wildlife diseases,* **33**: 812-817.

Borchers, K., Lieckfeldt, D., Ludwig, A., Fukushi, H., Allen, G., Fyumagwa, R. and Hoare, R. 2008. Detection of Equid herpesvirus 9 DNA in the trigeminal ganglia of a Burchell's zebra from the Serengeti ecosystem. *The Journal of veterinary medical science / the Japanese Society of Veterinary Science,* **70**: 1377-1381.

Borchers, K., Wiik, H., Frolich, K., Ludwig, H. and East, M.L. 2005. Antibodies against equine herpesviruses and equine arteritis virus in Burchell's zebras (Equus burchelli) from the Serengeti ecosystem. *Journal of wildlife diseases,* **41**: 80-86.

Borchers, K., Wolfinger, U., Lawrenz, B., Schellenbach, A. and Ludwig, H. 1997. Equine herpesvirus 4 DNA in trigeminal ganglia of naturally infected horses detected by direct in situ PCR. *The Journal of general virology,* **78 (Pt 5)**: 1109-1114.

Borchers, K., Wolfinger, U. and Ludwig, H. 1999. Latency-associated transcripts of equine herpesvirus type 4 in trigeminal ganglia of naturally infected horses. *The Journal of general virology,* **80 (Pt 8)**: 2165-2171.

Carvalho, R., Oliveira, A.M., Souza, A.M., Passos, L.M. and Martins, A.S. 2000. Prevalence of equine herpesvirus type 1 latency detected by polymerase chain reaction. *Arch Virol,* **145**: 1773-1787.

Chowdhury, S.I., Kubin, G. and Ludwig, H. 1986. Equine herpesvirus type 1 (EHV-1) induced abortions and paralysis in a Lipizzaner stud: a contribution to the classification of equine herpesviruses. *Arch Virol,* **90**: 273-288.

Chowdhury, S.I., Ludwig, H. and Buhk, H.J. 1988. Molecular biological characterization of equine herpesvirus type 1 (EHV-1) isolates from ruminant hosts. *Virus Res,* **11**: 127-139.

Crabb, B.S., MacPherson, C.M., Reubel, G.H., Browning, G.F., Studdert, M.J. and Drummer, H.E. 1995. A type-specific serological test to distinguish antibodies to equine herpesviruses 4 and 1. *Arch Virol,* **140**: 245-258.

Crabb, B.S., Nagesha, H.S. and Studdert, M.J. 1992. Identification of equine herpesvirus 4 glycoprotein G: a type-specific, secreted glycoprotein. *Virology,* **190**: 143-154.

Crabb, B.S. and Studdert, M.J. 1993. Epitopes of glycoprotein G of equine herpesviruses 4 and 1 located near the C termini elicit type-specific antibody responses in the natural host. *J Virol,* **67**: 6332-6338.

Crandell, R.A., Ichimura, H. and Kit, S. 1988. Isolation and comparative restriction endonuclease DNA fingerprinting of equine herpesvirus-1 from cattle. *American journal of veterinary research,* **49**: 1807-1813.

Crowhurst, F.A., Dickinson, G. and Burrows, R. 1981. An outbreak of paresis in mares and geldings associated with equid herpesvirus 1. *Vet Rec,* **109**: 527-528.

Damiani, A.M., de Vries, M., Reimers, G., Winkler, S. and Osterrieder, N. 2014. A severe equine herpesvirus type 1 (EHV-1) abortion outbreak caused by a neuropathogenic strain at a breeding farm in northern Germany. *Vet Microbiol,* **172**: 555-562.

Davison, A.J. 2007. Overview of classification. In: Arvin A, Campadelli-Fiume G, Mocarski E, et al., editors. Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis. Cambridge: Cambridge University Press. Chapter 1. Available from: http://www.ncbi.nlm.nih.gov/books/NBK47406.

Davison, A.J., Eberle, R., Ehlers, B., Hayward, G.S., McGeoch, D.J., Minson, A.C., Pellett, P.E., Roizman, B., Studdert, M.J. and Thiry, E. 2009. The order Herpesvirales. *Arch Virol,* **154**: 171-177.

Dimock, W. 1933. Is there a filterable virus of abortion in mares. *Edwards P. Ky Agric Exp Stn Bull.,* **333:291-301.**

Dunowska, M. 2014a. A review of equid herpesvirus 1 for the veterinary practitioner. Part A: clinical presentation, diagnosis and treatment. *N Z Vet J.,* **62(4):171-8. doi: 10.1080/00480169.2014.899945.**

Dunowska, M. 2014b. A review of equid herpesvirus 1 for the veterinary practitioner. Part B: pathogenesis and epidemiology. *N Z Vet J.,* **62(4):179-88. doi: 10.1080/00480169.2014.899946.**

Dutch, R.E., Bianchi, V. and Lehman, I.R. 1995. Herpes simplex virus type 1 DNA replication is specifically required for high-frequency homologous recombination between repeated sequences. *J Virol,* **69**: 3084-3089.

Edington, N., Bridges, C.G. and Huckle, A. 1985. Experimental reactivation of equid herpesvirus 1 (EHV 1) following the administration of corticosteroids. *Equine veterinary journal,* **17**: 369-372.

El-Habashi, N., El-Nahass el, S., Fukushi, H., Hibi, D., Sakai, H., Sasseville, V. and Yanai, T. 2010. Experimental intranasal infection of equine herpesvirus 9 (EHV-9) in suckling hamsters: kinetics of viral transmission and inflammation in the nasal cavity and brain. *J Neurovirol,* **16**: 242-248.

El-Habashi, N., Murakami, M., El-Nahass, E., Hibi, D., Sakai, H., Fukushi, H., Sasseville, V. and Yanai, T. 2011. Study on the infectivity of equine herpesvirus 9 (EHV-9) by different routes of inoculation in hamsters. *Veterinary pathology,* **48**: 558-564.

El-Nahass, E., El-Habashi, N., Nayel, M., Kasem, S., Fukushi, H., Suzuki, Y., Hirata, A., Sakai, H. and Yanai, T. 2011. Kinetics and pathogenicity of equine herpesvirus-9 infection following intraperitoneal inoculation in hamsters. *J Comp Pathol,* **145**: 271-281.

Elmore, D. and Eberle, R. 2008. Monkey B virus (Cercopithecine herpesvirus 1). *Comp Med,* **58**: 11-21.

Escalera-Zamudio, M., Zepeda-Mendoza, M.L., Loza-Rubio, E., Rojas-Anaya, E., Mendez-Ojeda, M.L., Arias, C.F. and Greenwood, A.D. 2015. The evolution of bat nucleic acid-sensing Toll-like receptors. *Molecular ecology,* **24**: 5899-5909.

Fischer-Tenhagen, C., Hamblin, C., Quandt, S. and Frolich, K. 2000. Serosurvey for selected infectious disease agents in free-ranging black and white rhinoceros in Africa. *Journal of wildlife diseases,* **36**: 316-323.

Fukushi, H., Taniguchi, A., Yasuda, K., Yanai, T., Masegi, T., Yamaguchi, T. and Hirai, K. 2000. A hamster model of equine herpesvirus 9 induced encephalitis. *J Neurovirol,* **6**: 314-319.

Fukushi, H., Tomita, T., Taniguchi, A., Ochiai, Y., Kirisawa, R., Matsumura, T., Yanai, T., Masegi, T., Yamaguchi, T. and Hirai, K. 1997. Gazelle herpesvirus 1: a new neurotropic herpesvirus immunologically related to equine herpesvirus 1. *Virology,* **227**: 34-44.

Fukushi, H., Yamaguchi, T. and Yamada, S. 2012. Complete genome sequence of equine herpesvirus type 9. *J Virol,* **86**: 13822.

Gardiner, D.W., Lunn, D.P., Goehring, L.S., Chiang, Y.W., Cook, C., Osterrieder, N., McCue, P., Del Piero, F., Hussey, S.B. and Hussey, G.S. 2012. Strain impact on equine herpesvirus type 1 (EHV-1) abortion models: viral loads in fetal and placental tissues and foals. *Vaccine,* **30**: 6564-6572.

Ghanem, Y.M., Fukushi, H., Ibrahim, E.S., Ohya, K., Yamaguchi, T. and Kennedy, M. 2008. Molecular phylogeny of equine herpesvirus 1 isolates from onager, zebra and Thomson's gazelle. *Arch Virol,* **153**: 2297-2302.

Gibson, J.S., Slater, J.D., Awan, A.R. and Field, H.J. 1992. Pathogenesis of equine herpesvirus-1 in specific pathogen-free foals: primary and secondary infections and reactivation. *Arch Virol,* **123**: 351-366.

Goodman, L.B., Loregian, A., Perkins, G.A., Nugent, J., Buckles, E.L., Mercorelli, B., Kydd, J.H., Palu, G., Smith, K.C., Osterrieder, N. and Davis-Poynter, N. 2007. A point mutation in a herpesvirus polymerase determines neuropathogenicity. *PLoS pathogens,* **3**: e160.

Greenwood, A.D., Tsangaras, K., Ho, S.Y., Szentiks, C.A., Nikolin, V.M., Ma, G., Damiani, A., East, M.L., Lawrenz, A., Hofer, H. and Osterrieder, N. 2012. A potentially fatal mix of herpes in zoos. *Curr Biol,* **22**: 1727-1731.

Guo, X., Izume, S., Okada, A., Ohya, K., Kimura, T. and Fukushi, H. 2014. Full genome sequences of zebra-borne equine herpesvirus type 1 isolated from zebra, onager and Thomson's gazelle. *The Journal of veterinary medical science / the Japanese Society of Veterinary Science,* **76**: 1309-1312.

Hebia-Fellah, I., Leaute, A., Fieni, F., Zientara, S., Imbert-Marcille, B.M., Besse, B., Fortier, G., Pronost, S., Miszczak, F., Ferry, B., Thorin, C., Pellerin, J.L. and Bruyas, J.F. 2009. Evaluation of the presence of equine viral herpesvirus 1 (EHV-1) and equine viral herpesvirus 4 (EHV-4) DNA in stallion semen using polymerase chain reaction (PCR). *Theriogenology,* **71**: 1381-1389.

Ibrahim, E.S., Kinoh, M., Matsumura, T., Kennedy, M., Allen, G.P., Yamaguchi, T. and Fukushi, H. 2007. Genetic relatedness and pathogenicity of equine herpesvirus 1 isolated from onager, zebra and gazelle. *Arch Virol,* **152**: 245-255.

Jackson, T.A., Osburn, B.I., Cordy, D.R. and Kendrick, J.W. 1977. Equine herpesvirus 1 infection of horses: studies on the experimentally induced neurologic disease. *American journal of veterinary research,* **38**: 709-719.

Javier, R.T., Sedarati, F. and Stevens, J.G. 1986. Two avirulent herpes simplex viruses generate lethal recombinants in vivo. *Science,* **234**: 746-748.

Kasem, S., Yamada, S., Kiupel, M., Woodruff, M., Ohya, K. and Fukushi, H. 2008. Equine herpesvirus type 9 in giraffe with encephalitis. *Emerg Infect Dis,* **14**: 1948-1949.

Kinyili, J.H. and Thorsen, J. 1979. Antigenic comparisons between herpesviruses isolated from fallow deer in Alberta and the viruses of infectious bovine rhinotracheitis, equine rhinopneumonitis and DN-599, a non-IBR bovine herpesvirus. *Journal of wildlife diseases,* **15**: 339-341.

Kodama, A., Yanai, T., Yomemaru, K., Sakai, H., Masegi, T., Yamada, S., Fukushi, H., Kuraishi, T., Hattori, S. and Kai, C. 2007. Acute neuropathogenicity with experimental infection of equine herpesvirus 9 in common marmosets (Callithrix jacchus). *J Med Primatol,* **36**: 335-342.

Kydd, J.H., Townsend, H.G. and Hannant, D. 2006. The equine immune response to equine herpesvirus-1: the virus and its vaccines. *Veterinary immunology and immunopathology,* **111**: 15-30.

Lang, A., de Vries, M., Feineis, S., Muller, E., Osterrieder, N. and Damiani, A.M. 2013. Development of a peptide ELISA for discrimination between serological responses to equine herpesvirus type 1 and 4. *Journal of virological methods*.

Lunn, D.P., Davis-Poynter, N., Flaminio, M.J., Horohov, D.W., Osterrieder, K., Pusterla, N. and Townsend, H.G. 2009. Equine herpesvirus-1 consensus statement. *Journal of veterinary internal medicine / American College of Veterinary Internal Medicine,* **23**: 450-461.

Ma, G., Azab, W. and Osterrieder, N. 2013. Equine herpesviruses type 1 (EHV-1) and 4 (EHV-4)--masters of co-evolution and a constant threat to equids and beyond. *Vet Microbiol,* **167**: 123-134.

Norberg, P. 2010. Divergence and genotyping of human alpha-herpesviruses: an overview. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases,* **10**: 14-25.

Pagamjav, O., Sakata, T., Matsumura, T., Yamaguchi, T. and Fukushi, H. 2005. Natural recombinant between equine herpesviruses 1 and 4 in the ICP4 gene. *Microbiology and immunology,* **49**: 167-179.

Patel, J.R., Edington, N. and Mumford, J.A. 1982. Variation in cellular tropism between isolates of equine herpesvirus-1 in foals. *Arch Virol,* **74**: 41-51.

Pusterla, N., Byrne, B.A., Hodzic, E., Mapes, S., Jang, S.S. and Magdesian, K.G. 2010a. Use of quantitative real-time PCR for the detection of Salmonella spp. in fecal samples from horses at a veterinary teaching hospital. *Veterinary journal,* **186**: 252-255.

Pusterla, N., Mapes, S. and David Wilson, W. 2012. Prevalence of latent alpha-herpesviruses in Thoroughbred racing horses. *Veterinary journal,* **193**: 579-582.

Pusterla, N., Mapes, S. and Wilson, W.D. 2010b. Prevalence of equine herpesvirus type 1 in trigeminal ganglia and submandibular lymph nodes of equids examined postmortem. *Vet Rec,* **167**: 376-378.

Pusterla, N., Wilson, W.D., Mapes, S., Finno, C., Isbell, D., Arthur, R.M. and Ferraro, G.L. 2009. Characterization of viral loads, strain and state of equine herpesvirus-1 using real-time PCR in horses following natural exposure at a racetrack in California. *Veterinary journal,* **179**: 230-239.

R.Göltenboth, W. Busch, J.H., A.Ochs and u, a.U.W. 1996. Herpesvirus infection in an Indian Tapir and a Black rhinoceros: Case report. *Proceed Amer Assoc Zoo Vets Puerto Vallarta, Mexiko*: 18-21.

Ramakrishnan, R., Poliani, P.L., Levine, M., Glorioso, J.C. and Fink, D.J. 1996. Detection of herpes simplex virus type 1 latency-associated transcript expression in trigeminal ganglia by in situ reverse transcriptase PCR. *J Virol,* **70**: 6519-6523.

Rebhun, W.C., Jenkins, D.H., Riis, R.C., Dill, S.G., Dubovi, E.J. and Torres, A. 1988. An epizootic of blindness and encephalitis associated with a herpesvirus indistinguishable from equine herpesvirus I in a herd of alpacas and llamas. *J Am Vet Med Assoc,* **192**: 953-956.

Reed, S.M. and Toribio, R.E. 2004. Equine herpesvirus 1 and 4. *Vet Clin North Am Equine Pract,* **20**: 631-642.

Salwa, A. 2004. A natural outbreak of Aujeszky's disease in farm animals. *Pol J Vet Sci,* **7**: 261-266.

Schrenzel, M.D., Tucker, T.A., Donovan, T.A., Busch, M.D., Wise, A.G., Maes, R.K. and Kiupel, M. 2008. New hosts for equine herpesvirus 9. *Emerg Infect Dis,* **14**: 1616-1619.

Slater, J. 2007. Equine herpesviruses. *In: Sellon DC, Long MT (eds). Equine Infectious Diseases.,* **Pp 134-53. Saunders Elsevier, St. Louis, Missouri, USA**.

Slater, J.D., Borchers, K., Thackray, A.M. and Field, H.J. 1994. The trigeminal ganglion is a location for equine herpesvirus 1 latency and reactivation in the horse. *The Journal of general virology,* **75 (Pt 8)**: 2007-2016.

Smith, D.J., Iqbal, J., Purewal, A., Hamblin, A.S. and Edington, N. 1998. In vitro reactivation of latent equid herpesvirus-1 from CD5+/CD8+ leukocytes indirectly by IL-2 or chorionic gonadotrophin. *The Journal of general virology,* **79 (Pt 12)**: 2997-3004.

Stamatakis, A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics,* **30**: 1312-1313.

Stierstorfer, B., Eichhorn, W., Schmahl, W., Brandmuller, C., Kaaden, O.R. and Neubauer, A. 2002. Equine herpesvirus type 1 (EHV-1) myeloencephalopathy: a case report. *Journal of veterinary medicine. B, Infectious diseases and veterinary public health,* **49**: 37-41.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular biology and evolution,* **24**: 1596-1599.

Taniguchi, A., Fukushi, H., Matsumura, T., Yanai, T., Masegi, T. and Hirai, K. 2000a. Pathogenicity of a new neurotropic equine herpesvirus 9 (gazelle herpesvirus 1) in horses. *The Journal of veterinary medical science / the Japanese Society of Veterinary Science,* **62**: 215-218.

Taniguchi, A., Fukushi, H., Yanai, T., Masegi, T., Yamaguchi, T. and Hirai, K. 2000b. Equine herpesvirus 9 induced lethal encephalomyelitis in experimentally infected goats. *Arch Virol,* **145**: 2619-2627.

Taylor, T.J. and Knipe, D.M. 2004. Proteomics of herpes simplex virus replication compartments: association of cellular DNA replication, repair, recombination, and chromatin remodeling proteins with ICP8. *J Virol,* **78**: 5856-5866.

Tearle, J.P., Smith, K.C., Platt, A.J., Hannant, D., Davis-Poynter, N.J. and Mumford, J.A. 2003. In vitro characterisation of high and low virulence isolates of equine herpesvirus-1 and -4. *Research in veterinary science,* **75**: 83-86.

Telford, E.A., Watson, M.S., McBride, K. and Davison, A.J. 1992. The DNA sequence of equine herpesvirus-1. *Virology,* **189**: 304-316.

Telford, E.A., Watson, M.S., Perry, J., Cullinane, A.A. and Davison, A.J. 1998. The DNA sequence of equine herpesvirus-4. *The Journal of general virology,* **79 (Pt 5)**: 1197-1203.

Thiry, E., Meurens, F., Muylkens, B., McVoy, M., Gogev, S., Thiry, J., Vanderplasschen, A., Epstein, A., Keil, G. and Schynts, F. 2005. Recombination in alphaherpesviruses. *Reviews in medical virology,* **15**: 89-103.

van der Meulen, K.M., Favoreel, H.W., Pensaert, M.B. and Nauwynck, H.J. 2006. Immune escape of equine herpesvirus 1 and other herpesviruses of veterinary importance. *Veterinary immunology and immunopathology,* **111**: 31-40.

van Maanen, C., Sloet van Oldruitenborgh-Oosterbaan, M.M., Damen, E.A. and Derksen, A.G. 2001. Neurological disease associated with EHV-1-infection in a riding school: clinical and virological characteristics. *Equine veterinary journal,* **33**: 191-196.

Walter, J., Balzer, H.J., Seeh, C., Fey, K., Bleul, U. and Osterrieder, N. 2012. Venereal shedding of equid herpesvirus-1 (EHV-1) in naturally infected stallions. *Journal of veterinary internal medicine / American College of Veterinary Internal Medicine,* **26**: 1500-1504.

Weissenbock, H., Hainfellner, J.A., Berger, J., Kasper, I. and Budka, H. 1997. Naturally occurring herpes simplex encephalitis in a domestic rabbit (Oryctolagus cuniculus). *Veterinary pathology,* **34**: 44-47.

Welch, H.M., Bridges, C.G., Lyon, A.M., Griffiths, L. and Edington, N. 1992. Latent equid herpesviruses 1 and 4: detection and distinction using the polymerase chain reaction and co-cultivation from lymphoid tissues. *The Journal of general virology,* **73 (Pt 2)**: 261-268.

Wohlsein, P., Lehmbecker, A., Spitzbarth, I., Algermissen, D., Baumgartner, W., Boer, M., Kummrow, M., Haas, L. and Grummer, B. 2011. Fatal epizootic equine herpesvirus 1 infections in new and unnatural hosts. *Vet Microbiol,* **149**: 456-460.

Wohlsein, P., Thiele, A., Fehr, M., Haas, L., Henneicke, K., Petzold, D.R. and Baumgartner, W. 2002. Spontaneous human herpes virus type 1 infection in a chinchilla (Chinchilla lanigera f. dom.). *Acta Neuropathol,* **104**: 674-678.

Wolff, P.L., Meehan, T.P., Basgall, E.J., Allen, G.P. and Sundberg, J.P. 1986. Abortion and perinatal foal mortality associated with equine herpesvirus type 1 in a herd of Grevy's zebra. *J Am Vet Med Assoc,* **189**: 1185-1186.

Yanai, T., Fujishima, N., Fukushi, H., Hirata, A., Sakai, H. and Masegi, T. 2003a. Experimental infection of equine herpesvirus 9 in dogs. *Veterinary pathology,* **40**: 263-267.

Yanai, T., Tujioka, S., Sakai, H., Fukushi, H., Hirai, K. and Masegi, T. 2003b. Experimental infection with equine herpesvirus 9 (EHV-9) in cats. *J Comp Pathol,* **128**: 113-118.

Yasunaga, S., Maeda, K., Matsumura, T., Kai, K., Iwata, H. and Inoue, T. 1998. Diagnosis and sero-epizootiology of equine herpesvirus type 1 and type 4 infections in Japan using a type-specific ELISA. *The Journal of veterinary medical science / the Japanese Society of Veterinary Science,* **60**: 1133-1137.

Chapter 2

Comprehensive serology based on a peptide ELISA to assess the prevalence of closely related equine herpesviruses in zoo and wild animals

Azza Abdelgawad[1], Robert Hermes[1], Armando Damiani[2], Benjamin Lamglait[3], Gábor Á. Czirják[1], Marion East[1], Ortwin Aschenborn[4], Christian Wenker[5], Samy Kasem[6], Nikolaus Osterrieder[2], and Alex D. Greenwood[1*]

[1]Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Strasse 17, 10315 Berlin, Germany.
[2]Institut für Virologie, Freie Universität Berlin, Robert-von-Ostertag-Str. 7-13, 14163 Berlin, Germany.
[3]Réserve Africaine de Sigean, 19 chemin du hameau du lac, 11130 Sigean, France.
[4]Bwabwata Ecological Institute, Susuwe, Zambezi Region, Namibia.
[5]Zoo Basel, Binningerstrasse 40, CH-4054 Basel, Switzerland.
[6]Virology Department, Faculty of Veterinary Medicine, Kafrelsheikh University, Elgeish street, 33516, Kafrelsheikh, Egypt.

PLoS One. 2015; 10(9), published Sept. 17, 2015
DOI: http://dx.doi.org/10.1371/journal.pone.0138370

Abstract

Equine herpesvirus type 1 (EHV-1) causes respiratory disorders and abortion in equids while EHV-1 regularly causes equine herpesvirus myeloencephalopathy (EHM), a stroke-like syndrome following endothelial cell infection in horses. Both EHV-1 and EHV-9 infections of non-definitive hosts often result in neuronal infection and high case fatality rates. Hence, EHV-1 and EHV-9 are somewhat unusual herpesviruses and lack strict host specificity, and the true extent of their host ranges have remained unclear. In order to determine the seroprevalence of EHV-1 and EHV-9, a sensitive and specific peptide-based ELISA was developed and applied to 428 sera from captive and wild animals representing 30 species in 12 families and five orders. Members of the *Equidae*, *Rhinocerotidae* and *Bovidae* were serologically positive for EHV-1 and EHV-9. The prevalence of EHV-1 in the sampled wild zebra populations was significantly higher than in zoos suggesting captivity may reduce exposure to EHV-1. Furthermore, the seroprevalence for EHV-1 was significantly higher than for EHV-9 in zebras. In contrast, EHV-9 antibody prevalence was high in captive and wild African rhinoceros species suggesting that they may serve as a reservoir or natural host for EHV-9. Thus, EHV-1 and EHV-9 have a broad host range favoring African herbivores

and may have acquired novel natural hosts in ecosystems where wild equids are common and are in close contact with other perissodactyls.

Introduction

The order Perissodactyla includes the three families *Equidae*, *Rhinocertidae*, and *Tapiridae*. In the *Equidae*, nine herpesviruses have been identified, six of which are allocated to the subfamily *Alphaherpesvirinae* and three in the subfamily *Gammaherpesvirinae*. EHV-1 is an alphaherpesvirus and arguably one of the most important equine pathogens with a worldwide distribution in domestic horses (*Equus ferus caballus*) in which it causes respiratory disease, abortion, neonatal death and neurological disorders [1]. Infections with EHV-1 or closely related viruses have been identified in other equids including zebras, domestic donkeys, and onagers [2-4]. Among non-equid perissodactyls, EHV-1 infection was reported in the Indian tapir (*Tapirus indicus)* and black rhinoceros *(Diceros bicornis)* [5, 6]. EHV-1 antibodies were detected with a prevalence of 8.8% in African white (*Ceratotherium simum*) and black (*Diceros bicornis*) rhinoceroses [5].

EHV-9, the most recently discovered equine alphaherpesvirus, was first described in captive Thomson's gazelle (*Gazelle thomsoni*) in Japan that suffered from neurological symptoms and died [7], but was considered an accidental host [8-10]. Neither the natural host nor the complete host range of EHV-9 is known, but EHV-9 causes lethal disease in a number of different species under experimental conditions [3, 7, 10-13]. Both EHV-1 and/or EHV-9 have been shown to infect species in captivity other than their known natural hosts, resulting in disease and fatality in non-perissodactyla species such as polar bear *(Ursus maritimus),* black bear *(U. americanus)*, llamas *(Lama glama)*, alpacas *(Vicugna pacos)*, blackbuck *(Antelopa cervicapra)*, Thomson's gazelle (*Gazelle thomsoni*) and giraffe (*Giraffa camelopardalis*) [7, 12, 14-18]. A recombinant zebra-EHV-1/EHV-9 infection was reported in a polar bear and in Indian rhinoceros (*Rhinoceros unicornis*), in both cases resulting in severe and ultimately fatal neurological symptoms [19, 20]. The complete host range of EHV-1 and EHV-9 and whether there are differences in captivity that potentially promote cross-species transmission remains unknown.

Serological studies conducted on free-living zebra populations (*Equus burcelli*) have demonstrated the presence of antibodies against EHV-1 and EHV-4 in South Africa and against EHV-9 in Tanzania where zebras share water sources and grazing areas with Thomson's gazelles and are thus frequently in close proximity to each other [3, 21, 22]. In Tanzania, the seroprevalence of EHV-1 and EHV-9 infections of 14% and 60%, respectively, was surveyed by serum neutralization test (SNT) [3]. However, equid alphaherpesviruses are

very similar genetically and antigenetically; thus, SNT's have poor discriminatory power for closely related viruses such as EHV-1, EHV-4 and EHV-9. Therefore, it is unlikely that SNT's allow discrimination between EHV-1 or EHV-9 antibodies [7, 10].

To accurately distinguish between the different virus strains, a type-specific-gG-based enzyme-linked immunosorbent assay (ELISA) was developed for EHV-1- and EHV-4-specific antibodies [23]. ELISAs were also developed to discriminate between EHV-1 and EHV-4 using peptides [glycoprotein E (gE) for EHV-1 and glycoprotein G (gG) for EHV-4] [24, 25]. However, assays to discriminate antibodies against EHV-1 and EHV-9 have not been developed.

In this study we developed and applied a peptide-based ELISA to detect and differentiate between EHV-1- and EHV-9-specific antibodies in serum of different species. Seroprevalence for both viruses was determined from 428 serum samples collected from captive and wild animals. The objectives of the study were to determine the prevalence of EHV-1 and EHV-9 infections and specifically to determine the host range in the wild and to identify equid and non-equid reservoir species.

Materials and methods

Serum samples

Sera were collected from non-vaccinated captive (n=277 samples from 43 zoos) and free ranging species (n=151 samples, Tanzania and Namibia) (Table 1). EHV-9 positive serum collected from an experimentally infected rabbit [26] was used as a positive control as no infected horse serum was available. An EHV-9 negative horse (umbilical cord blood serum) [24] sample was used as a negative control. An EHV-1-positive control serum collected at day 21 from an experimentally infected seronegative horse (horse a [24, 27]) and EHV-1-negative control (umbilical cord blood serum) collected from equine neonates immediately after birth [24] were used as controls for EHV-1 infection. Fetal calf serum was used as a negative control for SNT.

Table 1. List of animal species and number of serum samples used in this study.

	Zoo serum samples			
Order/***Family***/*species/ subspecies*		Number	Origin	Place of birth
Perissodactyla	**Odd-toed ungulates**			
Equidae			Captive	Captive
Equus quagga antiquorum	Damara zebra	6		
Equus quagga boehmi	Grant's zebra	32		
Equus quagga chapmani	Chapman's zebra	1		
Equus grevyi	Grévy's zebra	17		
Equus zebra hartmannae	Hartmann's mountain zebra	33		

Equus kiang	Kiang	3		
Equus ferus caballus	Pony	4		
Equus hemionus onager	Onager	5		
Equus africanus asinus	Donkey	12		
Equus africanus somalicus	Somali wild ass	19		
Rhinocerotidae			Captive	Captive
Ceratotherium simum	White rhinoceros	64		26 captive 36 wild 2 unknown
Diceros bicornis	Black rhinoceros	6		Captive
Artiodactyla	**Even-toed ungulates**		Captive	Captive
Bovidae				
Tragelaphus imberbis	Lesser kudu	13		
Hippotragus niger	Sable antelope	12		
Kobus megaceros	Nile lechwe	7		
Addax nasomaculatus	Addax antelope	2		
Taurotragus oryx	Common eland	2		
Ovibos moschatus	Muskox	1		
Giraffidae				
Giraffa camelopardalis	Giraffe	10		
Okapia johnstoni	Okapi	8		
Cervidae				
Alces alces alces	Moose	1		
Hippopotamidae				
Hippopotamus amphibious	Hippopotamus	1		
Carnivore			Captive	Captive
Ursidae				
Ursus maritimus	Polar bear	7		
Ursus thibetanus	Asian black bear	8		
Felidae				
Panthera leo krugeri	White lion	1		
Primates			Captive	Unknown
Cercopithecidae				
Papio hamadryas	Hamadryas baboon	1		
Erythrocebus patas	Patas monkey	1		
	Wild serum samples			
Perissodactyla			Wild	Wild
Equidae				
Equus quagga	Plains zebra	41	40 Tanzania 1 Namibia	
Rhinocerotidae				
Diceros bicornis	Black rhinoceros	17	Namibia	
Artiodactyla			Namibia	Wild
Bovidae				
Antidorcas marsupialis	Springbok	20		
Connochaetes gnou	Black wildebeest	22		
Carnivore			Namibia	Wild

Felidae				
Panthera leo	Lion	17		
Canidae				
Lycaon pictus	African wild dog	6		
Canis mesomelas	Black-backed jackal	10		
Hyaenidae				
Crocuta crocuta	Spotted hyaena	8		
Proboscidea			Namibia	Wild
Elephantidae				
Loxodonta africana	African elephant	10		

Ethics statement

The described research was approved by the Internal Ethics Committee of the Leibniz-institute for Zoo and Wildlife Research (IZW), Approval no. 2012-05-02.

Serum neutralization test

Serum neutralization test (SNT) was performed as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals with few modifications [3, 28]. Briefly, in 96-well micro plates, serial two fold dilutions of complement-inactivated serum samples at 56°C for 30 min were incubated with 100 plaque forming units (PFU)/100µl of EHV-1 or EHV-9 at 37°C. After 1 h incubation, 5×10^5 equine dermal cells (ED) were added and incubated for 2 h at 37°C. The wells were overlaid with 1.6% (w/v) methylcellulose medium and incubated at 37°C. The reaction was stopped after 4 days with 3% (v/v) formalin and the plaques were stained with Giemsa. EHV-1-positive horse serum and fetal calf serum were included as positive and negative controls, respectively. In the case of EHV-9, positive rabbit serum and fetal calf serum were included as positive and negative controls, respectively. SN antibody titers were calculated by determining the highest serum dilution that completely protects the monolayers from infection (no CPE) in each well. Titers of ≥ 1:4 were considered as positive. Each test was validated with the positive and negative sera controls. Each serum sample was tested twice in duplicate independently.

Peptides

Two sets of peptides, 18 amino acids (KQPQPRLRVKTPPPVTVP) for EHV-1_E (1E) and 15 amino acids (DSPPETPSPQENLND) for EHV-9_G (9G), that were biotinylated at the N-terminus and attached to two aminohexanoic acid hydrophobic spacers, to be able to attach to the streptavidin coated microtitre plates, were synthesized (Genscript, USA). The EHV-1_E peptide was used for descrimination between EHV-1 and EHV-4 as described previously [24]. EHV-9_G has shared 40% and 33% amino acid identity with gGs of EHV-1 horse strain (GenBank accession number: AET80923.1) and EHV-1 zebra strain (GenBank

accession number: AII81244.1), respectively and was used to differenciate between EHV-1 and -9.

An EHV-1_G peptide (ESSLENQLTQEESNN), was tested with EHV-1 and EHV-9-positive and negative controls to confirm the specificity of the selected EHV-9_G peptide (GenBank accession number: AII81244.1).

Peptide-based ELISA

The ELISA test was carried out on equids as described previously [24, 29] with few modifications. Briefly, 96-well plates (Sarstedt, Germany) were coated to 100 µl/well with 1µg/ml streptavidin dissolved in 50 nM carbonate-biocarbonate buffer (PH 9.6) overnight at 4°C. The wells were washed three times with PBS (PH 7.5) containing 0.1% (v/v) Tween 20 (PBST). After coating with 100 µl /well of the respective biotinylated peptide (2 µg/ml in 50 nM carbonate-biocarbonate buffer), the plates were incubated for 2 h at 37°C. Unoccupied sites were blocked by incubation for 1 h at 37°C with 1% (v/v) goat serum diluted in PBST. After washing, serum samples (100µl/well) were added in dilution of 1:400 and incubated for 1 h at 37°C. Purified goat anti-horse IgG conjugated with horseradish peroxidase (1:20000; Dianova, Germany), and HRP-goat anti-rabbit IgG for the EHV-9 positive control (1:10000; cell signaling, Germany) was added to each well. After 1 h incubation and washing, the plates were developed with 100 µl/well TMB [3,3,5,5-tetramethylbenzidine; dissolved in 42 µg/ml citric acid, 0.01% (v/v) H_2O_2 (pH 3.95)]. The reaction was stopped after 10 min with 100µl/ml of 1 M sulfuric acid and the plates were read at a wavelength of 450 nm on a spectrophotometer. Each serum sample was tested for significant EHV-1 and EHV-9 antibody titers at three independent times. Negative and positive serum controls were included in each plate.

Secondary antibody choice can be critical in serological analysis of different wildlife species [30]. For detection of EHV-1 and EHV-9 antibodies in different mammalian sera, the ELISA test was performed as described above except a 2% (w/v) albumin fraction was used as blocking buffer and protein G-peroxidase conjugate (1:10,000; Invitrogen, Germany) was used as a secondary antibody [30]. Protein G-peroxidase was shown to be capable of binding to the Fc region of immunoglobulin (IgG) without interfering with the antigen binding sites [30]. The protein G-peroxidase-dilution was optimized using 10 positive and 10 negative randomly selected zebra sera, based on our peptide-ELISA results shown. All negative samples yielded negative results with protein G-peroxidase and positive serum samples yielded the expected optical density (OD) values (S 1 Fig.). As controls, EHV-1 (wild zebra, PZ27) or EHV-9 (wild zebra, PZ34) positive zebra serum samples were used as a positive

control. For testing carnivore serum samples, recombinant HRP-labeled protein A/G (1:10000; Thermo scientific, USA) was used [30].

Assay sensitivity and specificity determination

The cutoff value is defined as the level of antibody activity which represents a minimum positive status for a tested animal. The method for determining a diagnostic cutoff is to test samples from known negative and positive populations [31]. In a previous study [24], the negative cutoff value for the EHV-1_E peptide was calculated based on data from 9 negative umbilical cord sera and 26 Icelandic horses sera, which were known to be immunologically naive to EHV-1. Due to the lack of specific pathogen free zebras and no EHV-1- or EHV-9-experimentally infected zebras, we used 27 zoo zebra sera (12 plains zebra, 9 Hartmann's mountain zebra, and 6 Grevy's zebra), which were free from detectable neutralizing antibodies against either EHV-1 or EHV-9 as tested by SNT (S1 Table), to calculate the negative cutoff value. A negative cutoff was calculated as the mean OD of zebra sera plus 2 SD and a positive cutoff was calculated as mean OD of this population plus 3 SD [24]. The cutoff values were applied to the non-equid samples as well because control serum and numbers of samples were insufficient to make an equivalent determination as with zebras and protein G peroxidase was shown not to interfere with the assay. The cutoff is conservative (mean OD plus or minus 2 to 3 SD) and it is more likely that some non-equids that were weakly positive were scored as negative than scoring animals as false positives.

Statistical analysis

All statistical analyses and graphs were performed using GraphPad Prism version 5.0a software (GraphPad Software, San Diego, CA). Fisher tests were used to compare the frequencies or proportions of EHV-1 and -9 positivity. Differences were considered statistically significant when the *P* value was less than 0.05.

Results

Peptide ELISA sensitivity and specificity

EHV-1 gE (EHV-1_E)- and EHV-9 gG (EHV-9_G)- peptides were used to differentiate between EHV-1- and EHV-9-specific antibodies. To test the reactivity and specificity of the selected EHV-9_G peptide, an EHV-9-positive rabbit serum and different positive and negative EHV-1 horse sera were used. The peptide produced high OD values (1.26) with an EHV-9-positive control serum and no reaction with EHV-1-positive (OD=0.06) or EHV-9-negative (OD=0.04) horse serum controls (Mann Whitney test, P=0.002; Fig. 1A).

The reactivity and specificity of the EHV-1_E peptide was previously determined [24]. The peptide produced high OD values (1.49) with an EHV-1-positive control, an OD value of 0.52 with the EHV-9-positive control and a low OD value (0.05) with EHV-1-negative controls. Although there was a significant difference between EHV-1- and EHV-9-positive controls (Mann Whitney test, P=0.002; Fig. 1B), this result did not unequivocally differentiate between EHV-1 and EHV-9 as the OD value of EHV-9-positive control was above the negative cutoff. An EHV-1_E peptide was used to detect EHV-1-positive antibodies, particularly in EHV-9-negative samples, and to exclude EHV-4 (a more distantly related virus to EHV-1 and EHV-9) infection [24].

To examine the specificity of the EHV-9_G peptide, EHV-1 and EHV-9 positive and negative controls were tested with a peptide with the EHV-1 gG sequence. The peptide produced OD values (0.4) with an EHV-1-positive control serum and OD=0.15 with EHV-9-positive control and no reaction with EHV-9-negative (OD=0.04) control (Mann Whitney test, P=0.02; Fig. 1C). Although it demonstrated a clear EHV-1 specificity, we did not rely on this peptide to discriminate the EHV-1 positive serum samples due to low reactivity with the EHV-1 positive control in comparison with the EHV-1_E peptide.

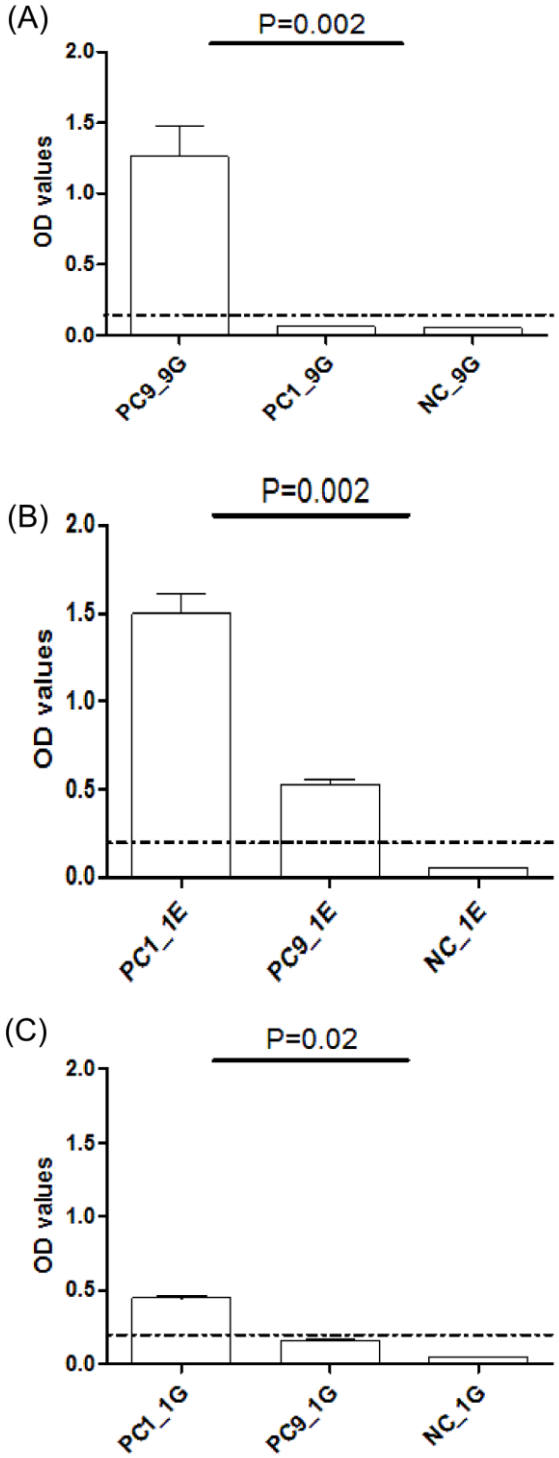


Fig 1. Reactivity and specificity of EHV peptides. Reactivity and specificity of (A) EHV-9_G (9G), (B) EHV-1_E (1E), and (C) EHV-1_G (1G) peptides using EHV-1 (PC1) (serum collected at day 21 from experimentally infected horse), EHV-9 (PC9) positive controls (rabbit serum) and NC (umbilical cord blood serum). The dashed line represents the negative cutoff value.

To determine the cutoff value, 27 zoo zebra sera were tested. The serum samples were considered EHV-1-positive when the OD value was higher than 0.2 and negative when the

OD value was lower than 0.18. For EHV-9, serum samples were considered positive when OD values exceed 0.14 and negative when below 0.13. OD values between 0.18 and 0.2 in case of EHV-1 and between 0.14 and 0.13 in case of EHV-9 were considered questionable and may represent antibody levels that are either very low (at the detection limit) or non-specific cross reactions (Fig. 2A and B). Three samples were tested positive for EHV-1, while one sample was tested positive for EHV-9.

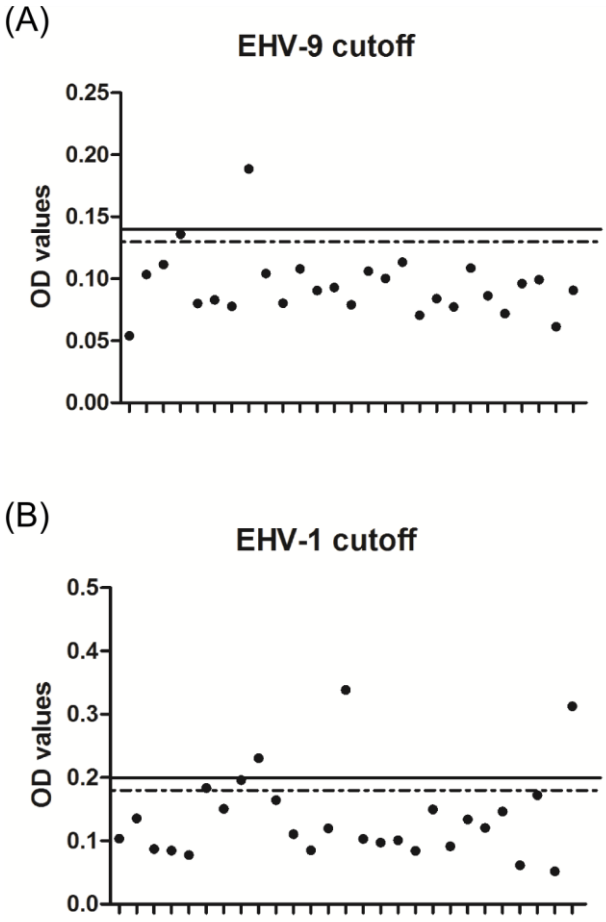


Fig 2. Determination of cutoff value. Twenty seven zebra serum samples were tested with either (A) EHV-9_G or EHV-1_E (B) peptides. OD values above the black line are considered positive, while OD values below the dashed line are considered negative. OD values between the lower and upper cut-offs were considered as questionable.

Detection of EHV-1 and EHV-9 neutralizing antibodies

None of the animals tested (Table 1), wild or captive, were noted to have displayed clinical symptoms including lesions associated with herpesvirus infection. Of 132 sera from equids in captivity, 60 and 59 were seropositive by SNT for EHV-1 and EHV-9, respectively (Table 2). In wild plains zebra serum samples (n=41), 32 and 35 tested positive for EHV-1

and EHV-9, respectively (Table 2). The samples collected from captive kiangs, onagers or ponies (n=12) were negative for antibodies against both viruses. Out of 64 captive white rhinoceros sera, 9 and 26 were positive by SNT for EHV-1 and EHV-9 antibodies, respectively. Captive black rhinoceros serum samples (n=6) did not show neutralizing antibodies against EHV-1 or EHV-9. In wild black rhinoceros, one and two out of 17 animals were positive for EHV-1 and -9 antibodies, respectively (Table 2). None of the tested captive or wild non-equid sera tested positive for either virus, except for one captive antelope [lesser kudu (*Tragelaphus imberbis*)], which had a low antibody titer for EHV-9 (Table 2). In the current study, neutralizing antibodies for either EHV-1 or EHV-9 were not detected in any of the tested carnivore serum samples (captive: n=16; wild: n=41; Table 1).

Table 2. Equine herpesvirus-antibody positive rates in captive and wildlife sera using SNT.

		Number (%) of positive results	
Family/ animal	Total	EHV-1	EHV-9
Captive sera			
Equidae			
Plains zebra	39	24 (61.5%)	21 (53.8%)
Grevy's zebra	17	9 (52.9%)	9 (52.9%)
Hartmann's mountain zebra	33	10 (30.3%)	15 (45.4%)
Donkey	12	4 (33.3%)	3 (27.2%)
Somali wild ass	19	13 (68.4%)	11 (57.8%)
Rhinocerotidae			
White rhinoceros	64	9 (14.0%)	26(40.6%)
Bovidae	37	0 (0.0%)	1 (2.7%)
Wild sera			
Equidae			
Plains zebra	41	32 (78%)	35 (85.3%)
Rhinocerotidae			
Black rhinoceros	17	1 (5.8%)	2 (11.7%)

Discrimination between EHV-1- and EHV-9- antibodies by peptide-based ELISA

All sera were tested by ELISA using EHV-1_E and EHV-9_G specific peptides (Table 1). In captive plains zebras, 22 and 10 serum samples tested positive for EHV-1 and EHV- 9 antibodies, respectively (n=39; Fig. 3A and B), 6 samples were classified as questionable for EHV-1, and one sample was questionable for EHV-9 antibodies. In wild plains zebras, 33 serum samples tested positive for EHV-1 exposure and 14 serum samples were positive for EHV-9 antibodies. The OD values in captive plains zebra were generally lower than those found in wild plains zebras (n=41; Fig. 3C and D). In captive Grevy's zebra, fewer (11 and 8 serum samples, respectively, were positive for EHV-1 and EHV-9 (n=17, Fig. 4A and B). In captive Hartman mountain zebra 22 and 8 samples were positive for EHV-

1 and EHV-9 antibodies, respectively (n=33, Fig. 5A and B). The data showed that the prevalence of EHV-1, but not EHV-9, antibodies in wild plains zebras was significantly higher than that in captive plains zebras (P=0.02; Fisher's exact test). There was no significant difference in EHV-1 (P=0.6; Fisher's exact test) and EHV-9 (P=0.2; Fisher's exact test) prevalence between the three tested captive zebra species.

Similar to the SNT results, onagers, ponies, and kiangs were negative for both EHV-1 and EHV-9 antibodies as detected with the ELISAs. Four donkeys tested positive (n=12) for EHV-1 antibodies. In Somali wild asses, 14 samples were positive for EHV-1 antibodies and one was positive for EHV-9 antibodies (n=19, Fig. 6A and B). The prevalence of EHV-1 antibodies in the tested plains zebra (wild or captive), captive Hartmann's zebra, and captive Somali wild ass sera was significantly higher than that of EHV-9 (P< 0.0001; Fisher's exact test).

Out of 64 captive white rhinoceros sera, 20 serum samples tested positive for EHV-1 exposure and 42 tested positive for EHV-9 antibodies (Fig. 7A and B). One of 6 tested captive black rhinoceroses' serum samples was positive for EHV-9 antibodies (Fig. 7C and D). In wild African black rhinoceros, 7 serum samples tested positive for EHV-1 exposure and 12 were positive for EHV-9 (n=17, Fig. 7E and F). The number of white rhinoceroses with exposure to EHV-9 was significantly higher than that with exposure to EHV-1 (P<0.05; Fisher's exact test). Although the prevalence of EHV-9 antibodies was higher than that of EHV-1 in black rhinoceros (either wild or captive), we could not detect a significance difference after statistical analysis, which may be due to the small sample size. Moreover, the prevalence of the detected EHV-9 antibodies was significantly higher in the complete tested rhinoceros population than the zebra population, in both captive and wild animals, (P<0.05; Fisher's exact test).

The only EHV-9-positive captive antelope serum sample tested positive by SNT was confirmed as positive by ELISA. In addition, two free-ranging springboks (*Antidorcas marsupialis*) were weakly positive for EHV-9 antibodies but that was only evident in the ELISA (Fig. 8).

None of the other animal families (*Giraffidae, Cervidae, Hippopotamidae, Cercopithecidae, Elephantidae*) tested positive for antibodies to either virus. We were unable to test for EHV-1 and EHV-9 antibodies in carnivores by ELISA (*Ursidae, Felidae, Canidae, Hyaenidae)* due to the high cross-reactivity of the protein A/G secondary antibody. The numbers of positive serum samples for each species are listed in details in Table 3.

Table 3. Equine herpesvirus-antibody positive rates in captive and wildlife sera using peptide-based ELISA.

Family/ animal	Total	Number (%) of positive results	
		EHV-1	EHV-9
Captive sera			
Equidae			
Plains zebra	39	22 (56.4%)	10 (25.6%)
Grevy's zebra	17	11 (64.7%)	8 (47%)
Hartmann's mountain zebra	33	22 (66.6%)	8 (24.2%)
Donkey	12	4 (33.3%)	0 (0.0%)
Somali wild ass	19	14 (73.6%)	1 (5.2%)
Rhinocerotidae			
White rhinoceros	64	20 (31.2%)	42 (65.6%)
Black rhinoceros	6	0	1 (16.6%)
Bovidae	37	0	1 (2.7%)
Wild sera			
Equidae			
Plains zebra	41	33 (80.4%)	14 (34.1%)
Rhinocerotidae			
Black rhinoceros	17	7 (41.1%)	12 (70.5%)
Bovidae	42	0 (0.0%)	2 (4.7%)

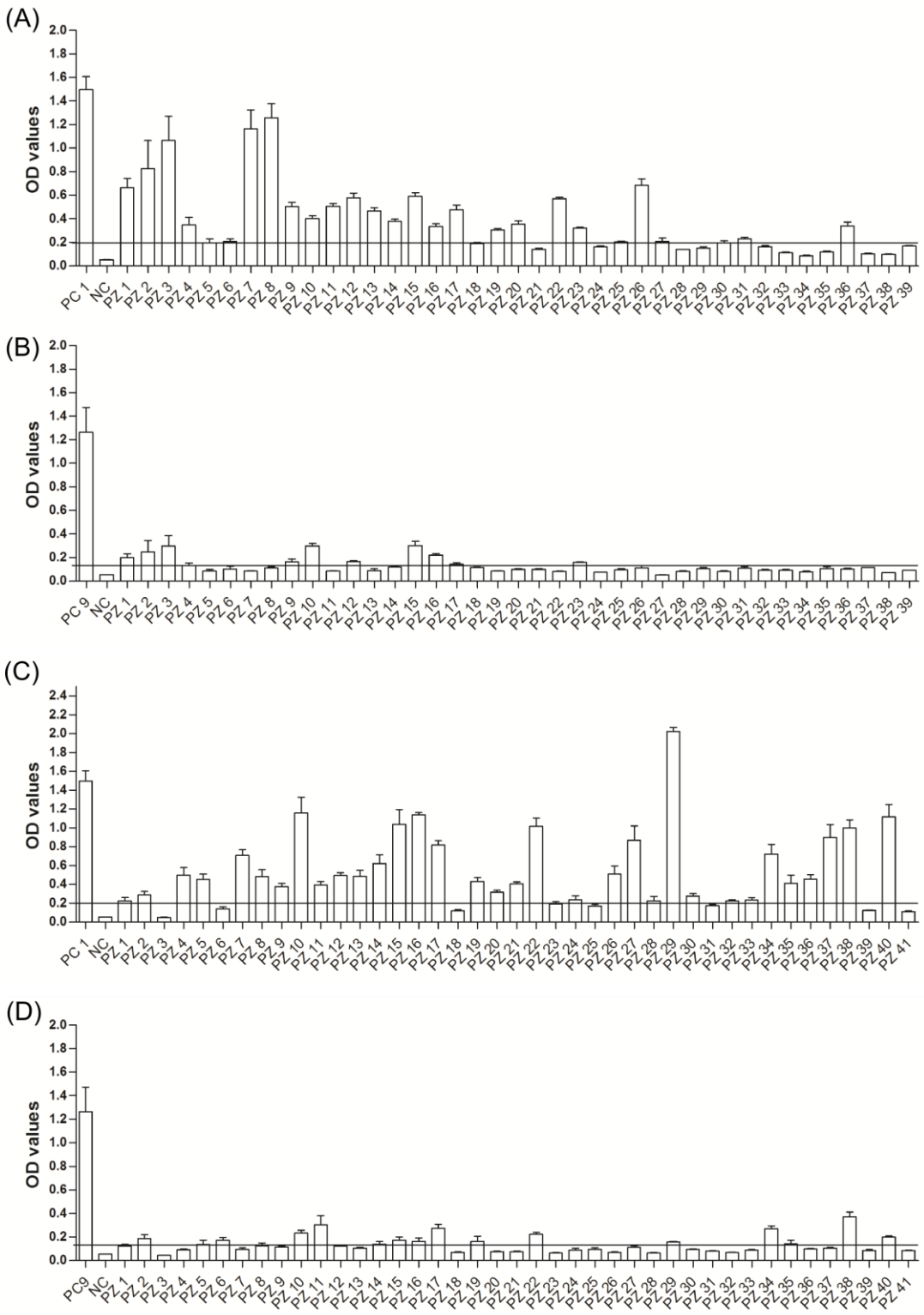


Fig 3. EHV antibodies in plains zebra. OD values of antibody responses tested by peptide-based ELISA in captive (A and B) and wild (C and D) plains zebra using EHV-1_E (A and C) and EHV-9_G (B and D) peptides. Black line: cutoff above which samples are considered positive. PC1= EHV-1 positive control (serum collected at day 21 from experimentally infected horse), PC9= EHV-9 positive control (serum collected from an experimentally infected rabbit), NC=negative control (umbilical cord blood serum), PZ= plains zebra.

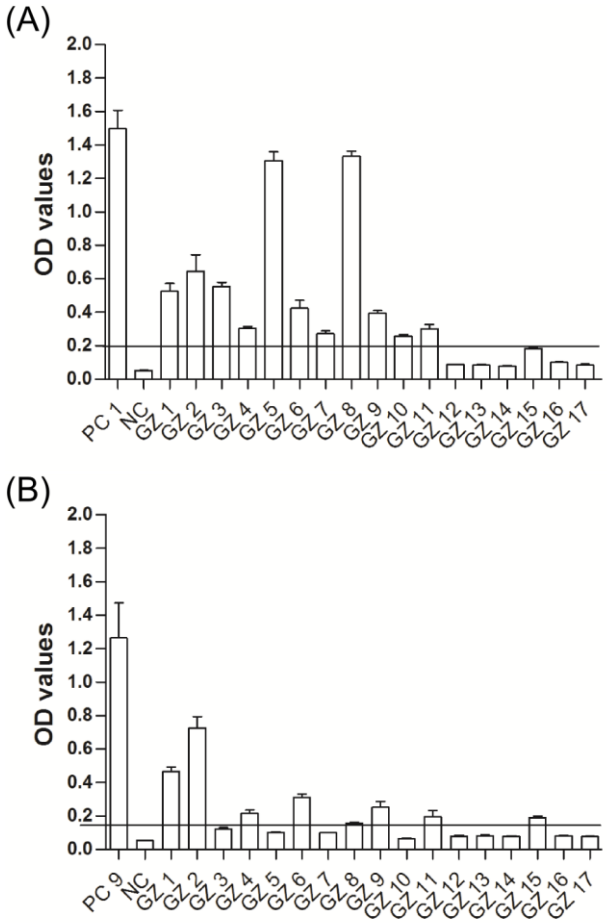


Fig 4. EHV antibodies in captive Grevy's zebra. OD values of antibody responses tested by peptide-based ELISA in captive Grevy's zebra using EHV-1_E (A) and EHV-9_G (B) peptides. Black line: cutoff above which samples are considered positive. PC1= EHV-1 positive control (serum collected at day 21 from experimentally infected horse), PC9= EHV-9 positive control (serum collected from an experimentally infected rabbit), NC=negative control (umbilical cord blood serum), GZ= Grevy's zebra.

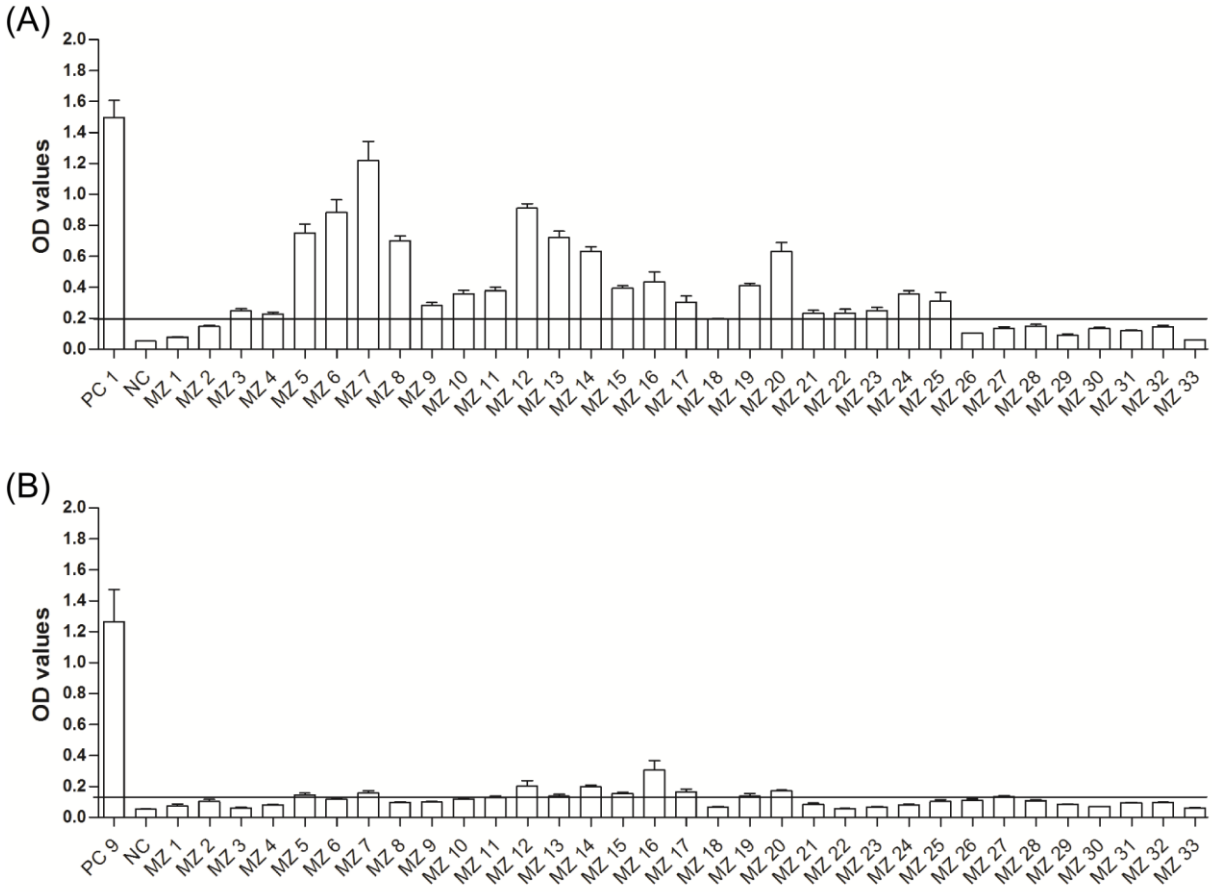


Fig 5. EHV antibodies in captive Hartmann's mountain zebra. OD values of antibody responses tested by peptide-based ELISA in captive Hartmann's mountain zebra using EHV-1_E (A) and EHV-9_G (B) peptides. Black line: cutoff above which samples are considered positive. PC1= EHV-1 positive control (serum collected at day 21 from experimentally infected horse), PC9= EHV-9 positive control (serum collected from an experimentally infected rabbit), NC=negative control (umbilical cord blood serum), MZ= Hartmann's mountain zebra.

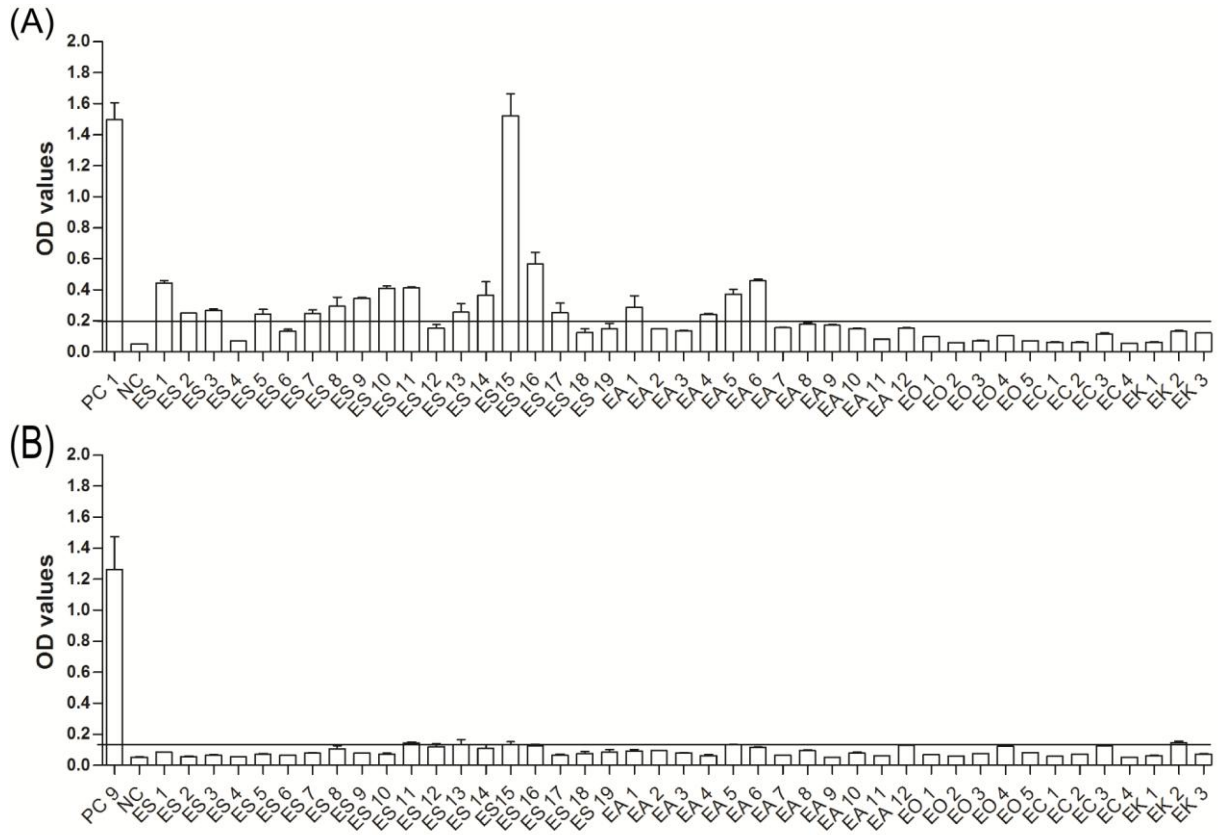


Fig 6. EHV antibodies in other captive equid species. OD values of antibody responses tested by peptide-based ELISA in other captive equid species using EHV-1_E (A) and EHV-9_G (B) peptides. Black line: cutoff above which samples are considered positive. PC1= EHV-1 positive control (serum collected at day 21 from experimentally infected horse), PC9= EHV-9 positive control (serum collected from an experimentally infected rabbit),, NC=negative control (umbilical cord blood serum), ES= *Equus africanus somalicus* (Somali wild ass), EA=*Equus africanus asinus* (donkey), EO=*Equus hemionus onager* (onager), EC=*Equus ferus caballus* (pony), EK=*Equus hemionus kiang* (kiang).

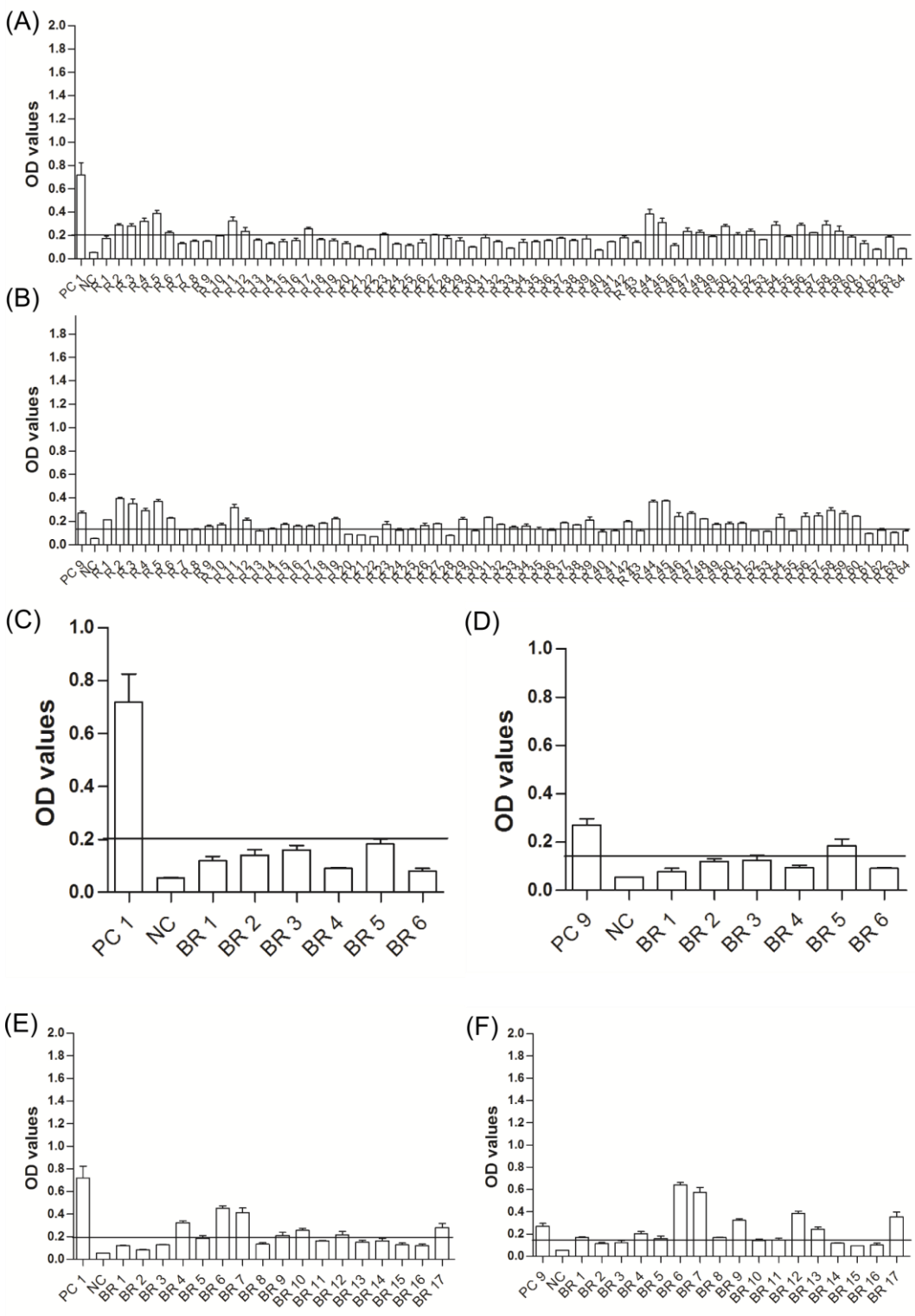


Fig 7. Prevalence of EHV-1 and EHV-9-antibodies in captive and wild rhinoceroses. OD values of antibody responses tested by peptide-based ELISA in captive white (A-B), black (C-D) and wild black (E-F) rhinoceroses using EHV-1_E (A, C, E) and EHV-9_G (B, D, F) peptides. Black line: cutoff above which samples are considered positive. PC= positive control ($PZ27^{W}$) for EHV-1 and ($PZ34^{W}$) for EHV-9, NC=negative control (umbilical cord blood serum), R= white rhino, BR= black rhino.

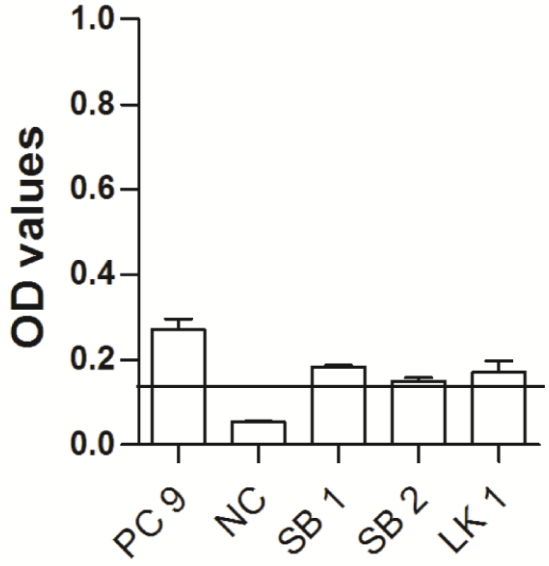


Fig 8. EHV antibodies in captive and wild antelopes. OD values of antibody responses tested by peptide-based ELISA in captive and wild antelope using EHV-9_G peptide. Black line: cutoff above which samples are considered positive. PC= positive control, PZ34^{w}, NC= negative control (umbilical cord blood serum), SB= springbok, LK= lesser kudu.

Molecular finding

Out of 17 wild African black rhinoceros samples, 3 blood samples were available in addition to serum. DNA was extracted and a nested PCR amplification of a partial sequence of the DNA-dependent-DNA polymerase gene was applied [20, 32]. A 250 bp fragment was amplified from one sample. Sanger sequencing revealed a sequence with 94% homology to the novel gammaherpesviruses sequence described from Damara's zebra (*E. burchellii antiquorum*), Somali wild ass (*E. asinus somalicus*), and eastern kiang (*E. kiang holdereri*) [12] (data not shown).

Discussion

None of the animals that tested positive for antibodies to either virus exhibited clinical symptoms indicative of herpesvirus infection. This is characteristic of natural viral host species and may be suggestive of co-adaptation of species that are natural conspecifics of equid reservoir animals. Serological analysis was undertaken because latency, the hallmark of herpesvirus infection, would probably underestimate the prevalence based on presence of viral antigen or nucleic acid. Furthermore, serological assays can be used for detection of possible reservoir hosts as described previously [33]. EHV-1 and EHV-9 share high nucleotide sequence similarity, the overall difference of their complete genome sequences being 9% [7]. As a result of this high degree of similarity, it is difficult to distinguish EHV-1

from EHV-9 serologically. SNT is known to be a sensitive and robust test, but may not accurately discriminate between similar viruses [34]. The limitations are well known for discrimination of EHV-1 and EHV-4, which share much less antigenic similarity compared to the EHV-1 and EHV-9 pair. Nonetheless, it is impossible to distinguish between EHV-1 and EHV-4 by SNT [24]. The epidemiological data of equine herpesviruses in zoo and wildlife is very limited and based on SNT [3, 21]. As an illustration of the problems associated with exclusive reliance on SNT, we found that 26 of 59 equid sera, which tested positive for EHV-9 by SNT, were negative by ELISA, results indicating that the detected serum neutralizing EHV-9 antibodies were a result of cross-reactivity with EHV-1. In contrast, peptide-ELISA has been successfully applied as a specific and sensitive serological test for detection of EHV-1 and EHV-4 seroprevalence [24]. A recent study also demonstrated that ELISA was more sensitive than SNT in detection of EHV-1 antibodies in milk [35]. ELISA tests, particularly when using strain specific peptides, are more discriminating and sensitive than SNT.

While the EHV-9_G peptide was highly specific and could accurately discriminate the EHV-9 infected animals, the EHV-1_E peptide cross reacted with EHV-9 positive samples. With all EHV-1-positive but EHV-9-negative serum samples, OD values considered positive were obtained with the EHV-1_E peptide, while OD values with the EHV-9_G peptide were negative. Many of the serum samples testing positive for EHV-9 were also positive for EHV-1. Whether this represents cross reaction or co-infection in the given serum samples is unclear. However, the EHV-1_E peptides allowed the exclusion of EHV-4 infection [24]. While this does present some limitations in determining the specific exposure status of the animals examined, the results provide a much higher resolution determination of infection history, particularly for EHV-9, than was previously possible for such genetically similar viral strains.

Sampling was undertaken with two goals in mind. The first was to sample as many zebras as possible, both captive and wild to determine the prevalence of EHV-1 and EHV-9 in the presumptive host for both viruses. The second goal was to sample African con-specifics of zebras as broadly as possible to determine the range of species potentially infected rather than focusing on any one species. Peptide-based ELISA demonstrated that the prevalence of EHV-1 antibodies in equids (wild or captive plains zebra, captive Hartmann's zebra, and captive Somali wild ass) was significantly higher than that of EHV-9. However, due to the cross reactivity of EHV-9 positive antibodies with the EHV-1_E peptide, further study will be needed to confirm the results obtained or refine the discriminatory power of the

ELISA. These results strongly suggest that these equid species are a natural and definitive host for EHV-1. There was no significant difference in EHV-1 and EHV-9 prevalence between the three tested zebra species. The results were not influenced by zebra species, thus all zebras exhibited very similar prevalence. While wild Hartmann's zebra and Somali wild ass sera were not available for this study, the lack of significant difference between wild and captive plains zebra and any plains zebra to the other equids suggests a similar prevalence could be expected in the wild for these species. EHV-1 and EHV-9 infections were reported previously in onager and pony [36, 37]; however, we could not detect antibodies for any of the viruses in these species, which might be due to the low sample size. In contrast, both EHV-1- and EHV-9-specific antibodies were detected in Somali wild asses. EHV-1 antibodies have been detected in Somali wild asses [18], however this is the first report of EHV-9 antibodies in this species. Recombination between EHV-1 and EHV-9 has been observed in sequences isolated from polar bear and Asian rhinoceros [19, 20]. The co-occurrence of these viruses in equids suggests that closely related equid alphaherpesviruses have natural opportunities to recombine and help explain the origin of recombinant isolates.

Using the developed peptide ELISA, an unexpectedly high prevalence of EHV-9 antibodies among captive and free-living African rhinoceros species was observed, the inverse of the observed prevalence in zebras, which exhibited higher EHV-1 antibody prevalence. However, EHV-1 antibodies were detected in larger number of rhinoceroses when compared to a previous study that relied exclusively on the immunofluorescence assay illustrating the higher sensitivity and specificity of the peptide based ELISA developed here [5]. The unexpectedly high prevalence of EHV-9 antibodies in rhinoceroses (particularly, captive white rhinoceros) suggests they are susceptible to EHV-9 infection and may serve as a natural and possibly definitive host or reservoir. The question of the natural reservoir and definitive host is particularly important for EHV-9 as the source of the many fatal infections of ungulates under natural conditions was not identified. Both EHV-1 and EHV-9 have been involved in fatal encephalitis cases in captive polar bears without proximity to equids [12, 19]. Rhinoceroses, which were not considered a potential source of infection, may have been involved in these unexplained transmission events. Similarly, a recent Asian rhinoceros fatality as a consequence of EHV-1 infection was suspected from a zebra source but may have derived from African rhinoceros [20]. Mammals in many African ecosystems congregate at water sources during periods of seasonal water shortage, which may explain transmission of viruses among perissodactyls and non-perissodactyls. For example, EHV-9 antibodies were detected in one captive lesser kudu and two wild springbok by SNT and/or

ELISA. The low prevalence of EHV-9-positive antelopes as well as the previous fatal EHV-9 infection [7] supports the hypothesis that these species are accidental hosts of the virus. In addition to the serological evidence, the detection of zebra isolated equine gammaherpesviral DNA in one wild black rhinoceros blood sample illustrates that transmission of EHVs does occur within African ecosystems. The general lower OD values observed for zebras and rhinoceros in captivity likely represents lower exposure, re-exposure and viral reactivation in captivity similar to the observe loss of pathogens in mice after generations of captivity [38].

Carnivores can be infected with EHV-1 and/ or EHV-9 either experimentally as in dogs and cats causing neurotropic encephalitis and death [39, 40] or naturally in captive polar bears which died after displaying severe nervous manifestations [12, 19]. Neither EHV-1 nor EHV-9 was detected in any carnivore sample in the current study by SNT. However, ELISA could not be applied due to non-specific cross reaction with protein A/G. Nonetheless, our findings suggest that carnivores are less frequently infected by EHV or fail to seroconvert and based on experimental infection, may be more likely to exhibit neurological symptoms when infection does occur. African carnivores, many of which prey on or scavenge equids may have evolved resistance to EHV as a result of the high risk of exposure to infection. Polar bears and non-African carnivores would not be expected to have evolved such resistance which may explain the relatively frequent observed fatal disease in species that are not naturally sympatric with African Perissodactyls.

Taken together, we propose that EHV-1 and EHV-9 have evolved a broad host range among African mammals including distantly related perissodactyls. The results presented here show that different families including *Equidae*, *Rhinocerotidae* and *Bovidae*, respond with robust antibody responses to EHV-1 and EHV-9 exposure. The high prevalence in the *Rhinoceroteridae* in particular, suggests that they may be a natural host and/or reservoir for EHV-9. Further study is needed to determine the role of these animals in EHV epidemiology in both captivity and the wild.

Acknowledgements

We thank Alexis Lecu, PARC Zoologique de Paris, France, Arne Lawrenz, Wuppertal Zoo, Germany, Christine Gohl, Münchner Tierpark Hellabrunn, Germany, Claudia Szentiks, IZW, Germany, Dorothée Ordonneau, CERZA Lisieux Zoo, France, Gabby Drake, Chester Zoo, UK, Gudrun Wibbelt, IZW, Germany, Günter Strauss, Tierpark Berlin, Germany, Harald Wiik, TAWIRI – Messerli Foundation Wildlife Veterinary Programme, Julia Gräger, Zoo Hannover, Germany, Katrin Baumgartner, Nuremberg Zoo, Germany, Martina Hoby, Zoo Basel, Switzerland, Tobias Witzens and Annika Krengel, Wilhelma der Zoologisch-

Botanische Garten, Germany and Ulrike Rademacher, EAZA Equid TAG for providing us with the samples. We also thank Bettina Wachter, Jörg Melzheimer, Maren de Vries and Sonja Heinrich for their support.

Competing Interests

Association with the Réserve Africaine de Sigean does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

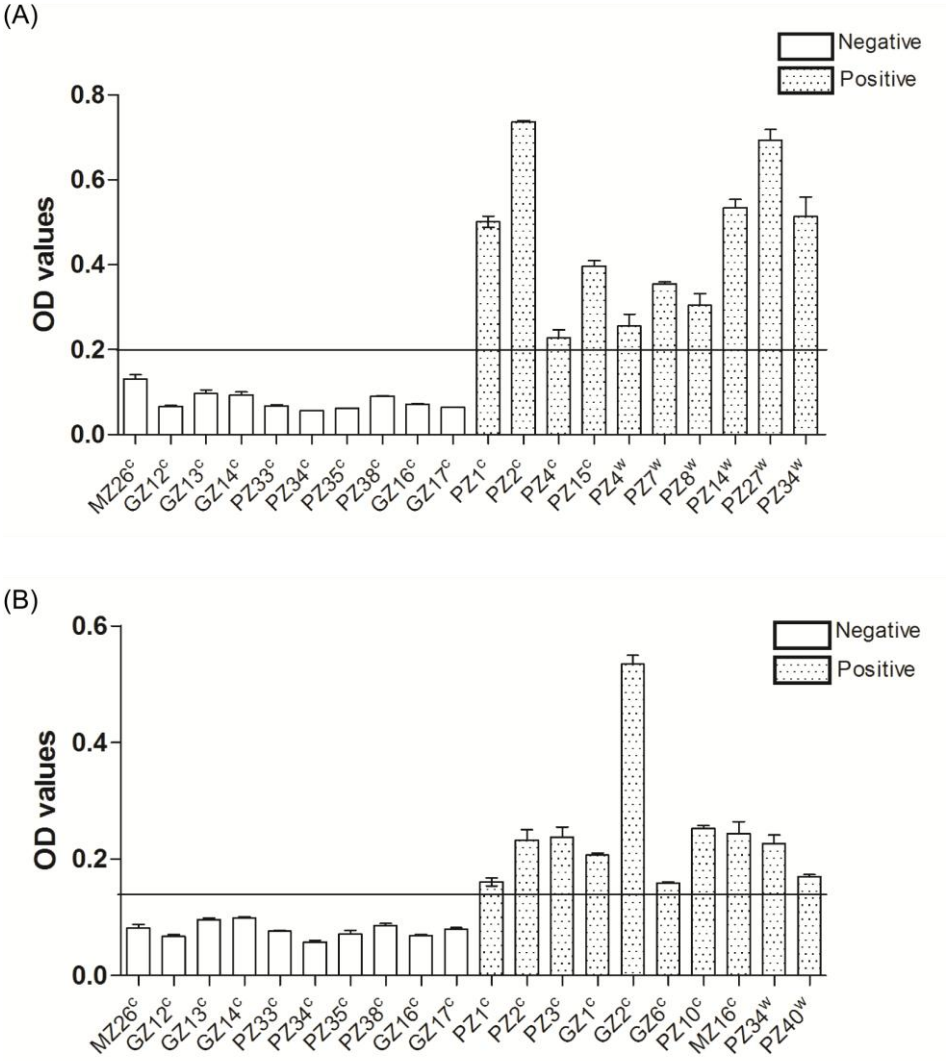
References

1. Reed SM, Toribio RE. Equine herpesvirus 1 and 4. Vet Clin North Am Equine Pract. 2004;20(3):631-42. doi: 10.1016/j.cveq.2004.09.001. PubMed PMID: 15519823.
2. Kydd JH, Townsend HG, Hannant D. The equine immune response to equine herpesvirus-1: the virus and its vaccines. Veterinary immunology and immunopathology. 2006;111(1-2):15-30. doi: 10.1016/j.vetimm.2006.01.005. PubMed PMID: 16476492.
3. Borchers K, Wiik H, Frolich K, Ludwig H, East ML. Antibodies against equine herpesviruses and equine arteritis virus in Burchell's zebras (Equus burchelli) from the Serengeti ecosystem. Journal of wildlife diseases. 2005;41(1):80-6. PubMed PMID: 15827213.
4. Damiani AM, de Vries M, Reimers G, Winkler S, Osterrieder N. A severe equine herpesvirus type 1 (EHV-1) abortion outbreak caused by a neuropathogenic strain at a breeding farm in northern Germany. Vet Microbiol. 2014;172(3-4):555-62. doi: 10.1016/j.vetmic.2014.06.023. PubMed PMID: 25042527.
5. Fischer-Tenhagen C, Hamblin C, Quandt S, Frolich K. Serosurvey for selected infectious disease agents in free-ranging black and white rhinoceros in Africa. Journal of wildlife diseases. 2000;36(2):316-23. PubMed PMID: 10813614.
6. R.Göltenboth, W. Busch JH, A.Ochs, u aUW. Herpesvirus infection in an Indian Tapir and a Black rhinoceros: Case report. Proceed Amer Assoc Zoo Vets Puerto Vallarta, Mexiko. 1996:18-21.
7. Fukushi H, Tomita T, Taniguchi A, Ochiai Y, Kirisawa R, Matsumura T, et al. Gazelle herpesvirus 1: a new neurotropic herpesvirus immunologically related to equine herpesvirus 1. Virology. 1997;227(1):34-44. PubMed PMID: 9015181.
8. Borchers K, Lieckfeldt D, Ludwig A, Fukushi H, Allen G, Fyumagwa R, et al. Detection of Equid herpesvirus 9 DNA in the trigeminal ganglia of a Burchell's zebra from the Serengeti ecosystem. The Journal of veterinary medical science / the Japanese Society of Veterinary Science. 2008;70(12):1377-81. PubMed PMID: 19122410.
9. Fukushi H, Taniguchi A, Yasuda K, Yanai T, Masegi T, Yamaguchi T, et al. A hamster model of equine herpesvirus 9 induced encephalitis. Journal of neurovirology. 2000;6(4):314-9. PubMed PMID: 10951555.
10. Taniguchi A, Fukushi H, Matsumura T, Yanai T, Masegi T, Hirai K. Pathogenicity of a new neurotropic equine herpesvirus 9 (gazelle herpesvirus 1) in horses. The Journal of veterinary medical science / the Japanese Society of Veterinary Science. 2000;62(2):215-8. PubMed PMID: 10720196.
11. Narita M, Uchimura A, Kimura K, Tanimura N, Yanai T, Masegi T, et al. Brain lesions and transmission of experimental equine herpesvirus type 9 in pigs. Veterinary pathology. 2000;37(5):476-9. PubMed PMID: 11055874.
12. Schrenzel MD, Tucker TA, Donovan TA, Busch MD, Wise AG, Maes RK, et al. New hosts for equine herpesvirus 9. Emerg Infect Dis. 2008;14(10):1616-9. PubMed PMID: 18826828.

13. Taniguchi A, Fukushi H, Yanai T, Masegi T, Yamaguchi T, Hirai K. Equine herpesvirus 9 induced lethal encephalomyelitis in experimentally infected goats. Arch Virol. 2000;145(12):2619-27. PubMed PMID: 11205108.
14. Chowdhury SI, Ludwig H, Buhk HJ. Molecular biological characterization of equine herpesvirus type 1 (EHV-1) isolates from ruminant hosts. Virus Res. 1988;11(2):127-39. PubMed PMID: 2849252.
15. Crandell RA, Ichimura H, Kit S. Isolation and comparative restriction endonuclease DNA fingerprinting of equine herpesvirus-1 from cattle. American journal of veterinary research. 1988;49(11):1807-13. PubMed PMID: 2854705.
16. Rebhun WC, Jenkins DH, Riis RC, Dill SG, Dubovi EJ, Torres A. An epizootic of blindness and encephalitis associated with a herpesvirus indistinguishable from equine herpesvirus I in a herd of alpacas and llamas. J Am Vet Med Assoc. 1988;192(7):953-6. PubMed PMID: 2835346.
17. Kasem S, Yamada S, Kiupel M, Woodruff M, Ohya K, Fukushi H. Equine herpesvirus type 9 in giraffe with encephalitis. Emerg Infect Dis. 2008;14(12):1948-9. PubMed PMID: 19046530.
18. Wohlsein P, Lehmbecker A, Spitzbarth I, Algermissen D, Baumgartner W, Boer M, et al. Fatal epizootic equine herpesvirus 1 infections in new and unnatural hosts. Vet Microbiol. 2011;149(3-4):456-60. PubMed PMID: 21167662.
19. Greenwood AD, Tsangaras K, Ho SY, Szentiks CA, Nikolin VM, Ma G, et al. A potentially fatal mix of herpes in zoos. Curr Biol. 2012;22(18):1727-31. PubMed PMID: 22902751.
20. Abdelgawad A, Azab W, Damiani AM, Baumgartner K, Will H, Osterrieder N, et al. Zebra-borne equine herpesvirus type 1 (EHV-1) infection in non-African captive mammals. Vet Microbiol. 2014;169(1-2):102-6. doi: 10.1016/j.vetmic.2013.12.011. PubMed PMID: 24440374.
21. Barnard BJ. Circulation of African horsesickness virus in zebra (Equus burchelli) in the Kruger National Park, South Africa, as measured by the prevalence of type specific antibodies. The Onderstepoort journal of veterinary research. 1993;60(2):111-7. PubMed PMID: 8332321.
22. Barnard BJ, Paweska JT. Prevalence of antibodies against some equine viruses in zebra (Zebra burchelli) in the Kruger National Park, 1991-1992. The Onderstepoort journal of veterinary research. 1993;60(3):175-9. PubMed PMID: 7970572.
23. Ma G, Azab W, Osterrieder N. Equine herpesviruses type 1 (EHV-1) and 4 (EHV-4)--masters of co-evolution and a constant threat to equids and beyond. Vet Microbiol. 2013;167(1-2):123-34. doi: 10.1016/j.vetmic.2013.06.018. PubMed PMID: 23890672.
24. Lang A, de Vries M, Feineis S, Muller E, Osterrieder N, Damiani AM. Development of a peptide ELISA for discrimination between serological responses to equine herpesvirus type 1 and 4. Journal of virological methods. 2013. doi: 10.1016/j.jviromet.2013.07.044. PubMed PMID: 23928223.
25. Yasunaga S, Maeda K, Matsumura T, Kai K, Iwata H, Inoue T. Diagnosis and sero-epizootiology of equine herpesvirus type 1 and type 4 infections in Japan using a type-specific ELISA. The Journal of veterinary medical science / the Japanese Society of Veterinary Science. 1998;60(10):1133-7. PubMed PMID: 9819768.
26. El-Nahass E, El-Habashi N, Abdelaziz AA, Nayel M, Kasem S, Fukushi H, et al. Kinetics and Pathogenicity of Oral Infection by Equine Herpesvirus-9 in Mice and Suckling Hamsters. J Comp Pathol. 2011. PubMed PMID: 21741053.
27. van Maanen C, Sloet van Oldruitenborgh-Oosterbaan MM, Damen EA, Derksen AG. Neurological disease associated with EHV-1-infection in a riding school: clinical and

virological characteristics. Equine veterinary journal. 2001;33(2):191-6. PubMed PMID: 11266070.
28. Office International des Epizooties 2008. Equine rhinopneumonitis. In Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Sixth Edition (Paris: Office International des Epizooties), pp. 894–903. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.05.09_EQUINE_RHINO.pdf
29. Crabb BS, Studdert MJ. Epitopes of glycoprotein G of equine herpesviruses 4 and 1 located near the C termini elicit type-specific antibody responses in the natural host. J Virol. 1993;67(10):6332-8. PubMed PMID: 7690425; PubMed Central PMCID: PMC238063.
30. Stobel K, Schonberg A, Staak C. A new non-species dependent ELISA for detection of antibodies to Borrelia burgdorferi s. l. in zoo animals. International journal of medical microbiology : IJMM. 2002;291 Suppl 33:88-99. PubMed PMID: 12141767.
31. Wright PF, Nilsson E, Van Rooij EM, Lelenta M, Jeggo MH. Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. Revue scientifique et technique. 1993;12(2):435-50. PubMed PMID: 8400384.
32. VanDevanter DR, Warrener P, Bennett L, Schultz ER, Coulter S, Garber RL, et al. Detection and analysis of diverse herpesviral species by consensus primer PCR. Journal of clinical microbiology. 1996;34(7):1666-71. PubMed PMID: 8784566; PubMed Central PMCID: PMC229091.
33. Fukushi H, Yamaguchi T, Yamada S. Complete genome sequence of equine herpesvirus type 9. J Virol. 2012;86(24):13822. doi: 10.1128/JVI.02607-12. PubMed PMID: 23166237; PubMed Central PMCID: PMC3503125.
34. Gilbert AT, Fooks AR, Hayman DT, Horton DL, Muller T, Plowright R, et al. Deciphering serology to understand the ecology of infectious diseases in wildlife. EcoHealth. 2013;10(3):298-313. doi: 10.1007/s10393-013-0856-0. PubMed PMID: 23918033.
35. R. Carvalho LMFP, A.M.G. Gouvea, M. Resende, A.S. Martins GCF. Use of an ELISA system for detection of equine herpesvirus 1 (EHV-1) antibodies in non-symptomatic pregnant mares and neonatal foals. Arq Bras Med Vet Zootec. 2000;vol.52 n.3.
36. Pusterla N, Wilson WD, Mapes S, Finno C, Isbell D, Arthur RM, et al. Characterization of viral loads, strain and state of equine herpesvirus-1 using real-time PCR in horses following natural exposure at a racetrack in California. Veterinary journal. 2009;179(2):230-9. doi: 10.1016/j.tvjl.2007.09.018. PubMed PMID: 18024200.
37. Ghanem YM, Fukushi H, Ibrahim ES, Ohya K, Yamaguchi T, Kennedy M. Molecular phylogeny of equine herpesvirus 1 isolates from onager, zebra and Thomson's gazelle. Arch Virol. 2008;153(12):2297-302. PubMed PMID: 19002555.
38. Gibson JS, Slater JD, Awan AR, Field HJ. Pathogenesis of equine herpesvirus-1 in specific pathogen-free foals: primary and secondary infections and reactivation. Arch Virol. 1992;123(3-4):351-66. PubMed PMID: 1314051.
39. Yanai T, Tujioka S, Sakai H, Fukushi H, Hirai K, Masegi T. Experimental infection with equine herpesvirus 9 (EHV-9) in cats. J Comp Pathol. 2003;128(2-3):113-8. PubMed PMID: 12634087.
40. Yanai T, Fujishima N, Fukushi H, Hirata A, Sakai H, Masegi T. Experimental infection of equine herpesvirus 9 in dogs. Veterinary pathology. 2003;40(3):263-7. PubMed PMID: 12724566.

Supporting Information



S1 Fig. Optimization of protein G-peroxidase. Ten negative and 10 positive zebra sera were tested with EHV-1_E (A) peptide and EHV-9_G (B) peptide. Black line: cutoff above which samples are considered positive. [c]=Captive serum sample, [w]=wild serum sample.

S 1 Table. SNT results of 27 zebra sera used to calculate the negative cutoff value.

Sample ID	Describtion	EHV-1 titer	EHV-9 titer
NC	Horse serum	<1:4	<1:4
PC		1:32	1:64
PZ29	plains zebra	<1:4	<1:4
PZ30	plains zebra	<1:4	<1:4
PZ31	plains zebra	<1:4	<1:4
PZ32	plains zebra	<1:4	<1:4
PZ33	plains zebra	<1:4	<1:4
PZ34	plains zebra	<1:4	<1:4
PZ35	plains zebra	<1:4	<1:4
PZ36	plains zebra	<1:4	<1:4
PZ37	plains zebra	<1:4	<1:4
PZ38	plains zebra	<1:4	<1:4
PZ39	plains zebra	<1:4	<1:4
PZ41	plains zebra	<1:4	<1:4
GZ12	Grevy's zebra	<1:4	<1:4
GZ13	Grevy's zebra	<1:4	<1:4
GZ14	Grevy's zebra	<1:4	<1:4
GZ15	Grevy's zebra	<1:4	<1:4
GZ16	Grevy's zebra	<1:4	<1:4
GZ17	Grevy's zebra	<1:4	<1:4
MZ25	Hartmann's mountain zebra	<1:4	<1:4
MZ26	Hartmann's mountain zebra	<1:4	<1:4
MZ27	Hartmann's mountain zebra	<1:4	<1:4
MZ28	Hartmann's mountain zebra	<1:4	<1:4
MZ29	Hartmann's mountain zebra	<1:4	<1:4
MZ30	Hartmann's mountain zebra	<1:4	<1:4
MZ31	Hartmann's mountain zebra	<1:4	<1:4
MZ32	Hartmann's mountain zebra	<1:4	<1:4
MZ33	Hartmann's mountain zebra	<1:4	<1:4

Chapter 3

Zebra alphaherpesviruses: genetic diversity, latency and co-infections

Azza Abdelgawad[a], Armando Damiani[b], Simon Y.W. Ho[c], Günter Strauss[d], Claudia A. Szentiks[a], Marion L. East[a], Nikolaus Osterrieder[b], and Alex D. Greenwood[a, e*]

[a]Leibniz-Institute for Zoo and Wildlife Research, Alfred-Kowalke-Strasse 17, 10315 Berlin, Germany

[b]Institut für Virologie, Freie Universität Berlin, Robert-von-Ostertag-Str. 7-13, 14163 Berlin, Germany

[c]School of Life and Environmental Sciences, University of Sydney, Sydney, NSW 2006, Australia

[d]Tierpark Berlin-Friedrichsfelde, Am Tierpark 125, 10307 Berlin, Germany

[e]Department of Veterinary Medicine, Freie Universität Berlin, Oertzenweg 19, 14163 Berlin, Germany

Abstract

Alphaherpesviruses are highly prevalent in equine populations and co-infections with more than one of these viruses' strains are frequently diagnosed. Lytic replication and latency with subsequent reactivation along with new episodes of disease can be influenced by genetic diversity generated by spontaneous mutation and recombination. Latency enhances virus survival by providing an epidemiological strategy for long-term maintenance of divergent strains in animal populations. The alphaherpesviruses equine herpesvirus 1 (EHV-1) and 9 (EHV-9) have recently been shown to cross species barriers, including a recombinant EHV-1 observed in fatal infections of a polar bear and Asian rhinoceros. Little is known about the latency and genetic diversity of EHV-1 and EHV-9, especially among zoo and wild equids. Here we report evidence of limited genetic diversity in EHV-9 in zebras, whereas there is substantial genetic variability in EHV-1. We demonstrate that zebras can be lytically and latently infected with both viruses concurrently. Such a co-occurrence of infection in zebras suggests that even relatively slow-evolving viruses such as equine herpesviruses have the potential to diversify rapidly by recombination. This has potential consequences for the diagnosis of these viruses and their management in wild and captive equid populations.

1. Introduction

Alphaherpesviruses are an ancient group of double-stranded DNA viruses that diverged from other herpesviruses ~200 million years ago (Thiry et al., 2005). Novel

alphaherpesviruses are frequently discovered and the extent of their genetic diversity is unknown. Differences in virulence and in biological and biochemical properties form an important basis for distinguishing between related herpesviruses, different virus strains and isolates (Thiry et al., 2005). Equine herpesviruses 1 (EHV-1) and 9 (EHV-9) are classified within the genus *Varicellovirus*, subfamily *Alphaherpesvirinae*, in the *Herpesviridae* family of the order *Herpesvirales* (Davison et al., 2009; Fukushi et al., 2012). EHV-1 and EHV-9 are unusual among herpesviruses, which are generally considered species-specific, due to their ability to infect species other than their natural hosts (Schrenzel et al., 2008; Wohlsein et al., 2011). Infections of non-definitive hosts can cause histopathological changes ranging from non-suppurative encephalitis to neuronal degeneration. Viral DNA has been detected in cases with high fatality rates in these hosts (Fukushi et al., 1997; Kasem et al., 2008; Schrenzel et al., 2008; Wohlsein et al., 2011).

We recently conducted a large-scale seroprevalence survey using a discriminatory specific peptide-based ELISA, with the aim of determining the host range and the possible reservoirs for EHV-1 and EHV-9 among zoo and wild animals. EHV-1 and EHV-9 exhibit a broad host range across mammalian families, including members of the Equidae, Rhinocerotidae and Bovidae, with a high prevalence of EHV-1 and EHV-9 antibodies in different geographic areas and habitats (Abdelgawad et al., 2015). Serological analysis was undertaken to avoid the underestimation of prevalence by relying on viral antigen or nucleic acid and, thus, the study did not provide information about the general genetic diversity of the circulation of EHVs in these populations. Little is known about the genetic characterization and diversity of EHV-1 and EHV-9 isolates circulating in zoo animals and wildlife. Studies suggest that zebras are the natural host for EHV-9, although serological prevalence also points to African rhinoceroses as an additional potential natural host (Abdelgawad et al., 2015; Borchers and Frolich, 1997; Borchers et al., 2008; Schrenzel et al., 2008).

The full genome of a single isolate of an EHV-1-zebra strain has been sequenced from Grevy's zebra (Guo et al., 2014). Much less genetic information is available for EHV-9 (Borchers et al., 2008; Norberg, 2010; Schrenzel et al., 2008). Therefore, the extent of strain divergence in wild and captive zebra populations is unknown. Considering that novel divergent EHVs have been associated with disease and fatalities among zoological collections, determining the genetic diversity and potential virulence of different strains is critical to the management of wild-derived equids in such collections.

Viruses from the subfamily *Alphaherpesvirinae*, including EHV-1, have the ability to establish a lifelong latent state in neurons of the sensory ganglia or in lymphoid cells, likely

cells of the monocytic and T-cell lineages in the case of EHV-1 (Aleman et al., 2012; Pusterla et al., 2009; Smith et al., 1998). The expression of lytic viral genes is repressed in latency and only a limited portion of the genome, usually comprising the so-called latency-associated transcripts (LATs) is expressed during the quiescent phase of infection (Ramakrishnan et al., 1996). LATs have been shown to be transcribed from the DNA strand opposite to that encoding ORF63 (the *ICP0* homolog) mRNA (Baxi et al., 1995; Pusterla et al., 2009; Ramakrishnan et al., 1996; Telford et al., 1998). The ability of herpesviruses to establish lytic and subsequent latent infection is an important epidemiological advantage for the virus to evade the host immune system during the latent phase and to establish a persistent infection (Dunowska, 2014; Welch et al., 1992). There is indirect evidence of EHV-9 latent infection in the trigeminal ganglion of free-living Burchell's zebras (Borchers et al., 2008).

Recombination and reassortment are mechanisms that are widely adopted by RNA viruses to increase their diversity. This is particularly pronounced in influenza viruses and retroviruses (Stamatakis, 2014). Genetic recombination has also been reported among alphaherpesviruses of high genetic similarity when infecting the same horse host at the same time (Norberg et al., 2015; Thiry et al., 2005). Natural recombination in the *ICP4* gene between different virus species such as EHV-1 and EHV-4 has been observed in horses (Pagamjav et al., 2005). A recent study of EHV-1 and EHV-4 isolates from horses in Australia and New Zealand demonstrated widespread recombination between EHV-1 and EHV-4, in particular (Norberg et al., 2004). Among EHVs isolated from wild equids, infection with a recombinant zebra-EHV-1/EHV-9 strain was reported in a polar bear and in an Indian rhinoceros (Abdelgawad et al., 2014; Greenwood et al., 2012). Whether recombination occurred in zebras in Africa or zoos elsewhere is unclear. However, viral co-infection is necessary for interspecific recombination in herpesviruses (Thiry et al., 2005).

Here we report both lytic and latent EHV-9 and EHV-1 infections in zoo and wild zebras and evaluate the relative genetic diversity in these viruses. Our study demonstrates, for the first time, an incidence of co-infection of EHV-9 and EHV-1 in a zebra. Our results collectively demonstrate that zebras can serve as a natural host for both viruses, with their co-occurrence suggesting an origin for recombinant zebra EHV strains.

2. Material and methods

2.1. Sample collection

Samples were obtained from 17 zoo equid animals: 14 animals necropsied at the Leibniz Institute for Zoo and Wildlife Research (Berlin, Germany) between 2012 and 2015, two animals from Zoo Hannover (Germany) and one animal from Réserve Africaine de

Sigean (France) (Supplementary Table 1). Tissues were collected from different organs, focusing on respiratory and nervous tissues. Respiratory lymph nodes and both left and right trigeminal ganglia were collected from ten animals to investigate viral latency. Sampling of trigeminal ganglia and lymph nodes was not possible from the other seven animals. In addition, brain tissues were opportunistically collected from carcasses of seven free-ranging plains zebras (Tanzania) killed by predators. The samples were stored immediately in RNA-later at -80ºC.

2.2. Histopathology

For histology investigations, specimens from different organs, lung, liver, heart, spleen, lymph nodes, olfactory bulb, and brain were collected and fixed in 4% neutral buffered formalin. Fixed tissue samples were routinely embedded in paraffin wax post-necropsy and serially sectioned at 4 µm and stained with haematoxylin and eosin (HE).

2.3. DNA and RNA extraction

Viral DNA was extracted from tissues using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (Klieforth et al., 2002). Total RNA was extracted from lymph nodes and trigeminal ganglia using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). For elimination of genomic DNA (gDNA), RNA was processed with the RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Before complementary DNA (cDNA) synthesis, RNA extraction from all samples was tested for gDNA absence using the housekeeping gene β-glucuronidase as a marker. Samples that were gDNA-free were further processed for cDNA synthesis, while positive samples were re-treated with RNase-Free DNase kit and re-tested. Subsequently, 1 µg of RNA was reverse-transcribed to cDNA using Superscript III (Life Technologies) and either random hexamer primers or a LAT-specific primer for *gB* and LAT, respectively (Pandey et al., 2015). Precautions were taken to avoid laboratory contamination during sample processing, including the use of disposable labware and separate facilities for DNA, RNA isolation and PCR amplification. Positive EHV-1 DNA, free DNA and RNA water, and RNA and DNA extracted from swabs from the work areas (NC swabs) were used as positive and negative controls.

2.4. Molecular characterization

PCR reactions using MyTaq HS polymerase mix (Bioline, Germany), 200 nM primers, and approximately 200 ng of the extracted template DNA were performed (Abdelgawad et al., 2014). Screening of herpesviruses using nested PCR targeting a partial sequence of the DNA-dependent-DNA polymerase gene (*Pol*, *UL30*) was performed as

described previously (VanDevanter et al., 1996). Amplification and sequencing of the *Pol*, *gB*, *UL49.5*, *ORF15* and genes encoded in the unique short segment (*ORF69–ORF74*; protein kinase, *gG*, *gp2*, *gD*, *gI*, and *gE*) was performed using primers specific to EHV-1 and EHV-9. The amplified products were purified and directly sequenced. The primers for nested PCR amplification, *ORF15* and *UL49.5* have been described previously (Abdelgawad et al., 2014). New primers used in this study are listed in Supplementary Table 2. The nucleotide sequences obtained in this study have been deposited in GenBank (accession numbers KX101085- KX101111).

2.5. Quantitative real-time PCR

For detection of latent infection in the collected lymph nodes and trigeminal ganglia, all samples were analyzed by quantitative real-time PCR (RT-qPCR) with the Applied Biosystems 7500 FAST (ABI, Foster City, CA, USA) to detect the presence of late *gB* gene and LATs based on *ORF63*. Primers and probes targeting a 90 bp product of *gB* were used as previously described (Pusterla et al., 2009). Specific primers and probes were designed to target 91 bp of *ORF63* (Genscript, USA); cDNA using specific primers for LAT RNA was used as a template. The TaqMan probe was labeled with 6-carboxyfluorescein (6-FAM) and the quencher carboxytetramethylrhodamine (TAMRA) at the 5' and 3' ends, respectively. The primers shared 100% similarity with EHV-1 and 96–100% similarity with EHV-9.

The qPCR protocol included an initial 95°C step for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. The qPCR reaction was performed in 20 µL using 1x SensiFAST™ Probe Lo-ROX Kit (Bioline, Germany), 450 nM of each primer, 100 µM of the respective TaqMan probe and 5 µL of the template. Standard curves created in this study were quantified by measuring absorbance at 260 / 280 nm using Nanodrop and an online application for calculating DNA copy number (http://endmemo.com/bio/dnacopynum.php). Amplification efficiency was calculated from the slope of a standard curve generated from 10-fold dilutions of EHV-1 genomic DNA for *gB* (Goodman et al., 2007) and synthetic oligonucleotides of the targeted region of *ORF63* (Supplementary Fig. 1). Assay sensitivity was assessed as described previously (D'Haene et al., 2010). DNA and RNA free-water and NC swabs were used as negative controls with each run. DNA and mRNA quantification for the *gB* and LATs, respectively, were compared with standard curves generated for each gene. The data were then normalized to a standard curve generated with oligonucleotides specific to equine beta-2-microglobulin (*B2M*). Viral DNA concentration was expressed as copies per million cells, considering that each diploid eukaryotic cell has two copies of the *B2M* gene (Allen, 2004; GP, 2004; Pusterla et al., 2009). For absolute quantification of the LAT, the

raw LAT cDNA data were normalized to the standard curve and then normalized to the calculated cell number based on the *B2M* gene data.

2.6. Genetic diversity and phylogenetic analyses

To estimate the mean nucleotide diversity (π), along with the average number of nucleotide substitutions per site within the EHV-1 and EHV-9 sequence groups, maximum composite likelihood model implemented in MEGA 7.0 with a gamma distribution for rate variation among sites was conducted (Nei and Jin, 1989; Tamura et al., 2007). The percentage of nucleotide substitutions per site between sequences was adapted in Tamura-Nei distances, while the standard error (SE) was estimated with 500 bootstrap replicates (Escalera-Zamudio et al., 2015).

To infer evolutionary relationships, we performed phylogenetic analyses of the nucleotide sequences of *gB*, *Pol* and *UL49.5* genes isolated from zebra tissues. Reference sequences for the same regions of EHV-1, EHV- 9, and EHV-4 were obtained from GenBank and aligned using ClustalW (Thompson et al., 1994). Phylogenetic trees were inferred using maximum-likelihood in RAxML v8.2.4 (Stamatakis, 2014) and Bayesian inference in MrBayes v3.2.2 (Ronquist et al., 2012). In the maximum-likelihood analyses, we performed a rapid bootstrapping analysis followed by a thorough search for the best-scoring tree. All trees were rooted with the EHV-4 sequences. In the Bayesian phylogenetic analysis, the GTR+G substitution model was used and a flat tree prior was specified. The posterior distributions of parameters, including the tree, were estimated using Markov chain Monte Carlo sampling. Samples were drawn every 1000 steps from a total of 10,000,000 steps. All analyses were run with one cold and one heated chain. The first 10% of samples were discarded as burn-in.

3. Results

3.1. Detection of herpesvirus infection

DNA was extracted from tissue samples collected from wild and zoo zebras and investigated for the presence of equine herpesviruses using a panherpes nested PCR. EHV-9 DNA was amplified from the nasal epithelium and/or olfactory bulb of two captive Grevy's zebras (CG2 and CG3) and brain tissue of one wild plains zebra (WP1). EHV-1 DNA was detected in the tissues of nasal epithelium and olfactory bulb of one captive plains zebra (CP4). Sequencing of the 250-bp fragment obtained from the nested PCR revealed 99% identity to previously reported sequences from the DNA polymerase (*Pol*) gene of EHV-1 and EHV-9. Sample abbreviations and infectious status of the tested samples are shown in Table 1.

PCR assays specific to EHV-1 and EHV-9 were next applied to target complete coding sequences of *gB* (2,643bp), *Pol* (3,660 bp) and *UL49.5* (400 bp; 300 *UL49.5* and 140 *UL50*) genes. In addition, partial coding sequences of *ORF15* and 9 kbp of the unique short region (*ORF69-ORF74*) were amplified from the positive wild and captive zebras. BLAST analysis followed by further alignment of the obtained sequences with different EHV-1 and EHV-9 sequences from NCBI revealed that WP1, CG2, and CG3 were infected with EHV-9, which showed high similarity to EHV-9 isolates detected in gazelle and giraffe. The 663 bp, 855 bp, and 411 bp of *gB*, *Pol*, and *UL49.5* genes, respectively, obtained from CP4 shared 99% similarity with EHV-1 isolates from horses.

3.2. Pathological findings

Gross lesions in respiratory or nervous tissue were not observed at necropsy. The clinical observations for the EHV-positive animals are described in Table 1. The captive zebra (CP4) exhibited ataxia and tremors without improvement after a course of medication. Microscopic examination revealed diffuse alveolar edema in lung tissue. The EHV-9-positive zebra (CG3) exhibited central nervous disorders with ataxia and restlessness; the animal collapsed and was subsequently euthanized. Non-suppurative encephalitis associated with perivascular cuffing in the internal granular layer (IV) of the frontal part of the cerebral cortex and gray matter of the cerebral cortex was observed (Fig. 1A). Meningeal perivascular inflammation with mainly lymphocytes and macrophages was also observed (Fig. 1B). Mild neuronal degeneration and glial reactions were found in the central nervous system, mainly in the cerebrum and olfactory bulb, but not in the cerebellum (Fig. 2A and B). Intra-nuclear inclusion bodies were not detected. Additionally, over-dilatation of lung alveoli with congestion in intra alveolar blood vessels was observed.

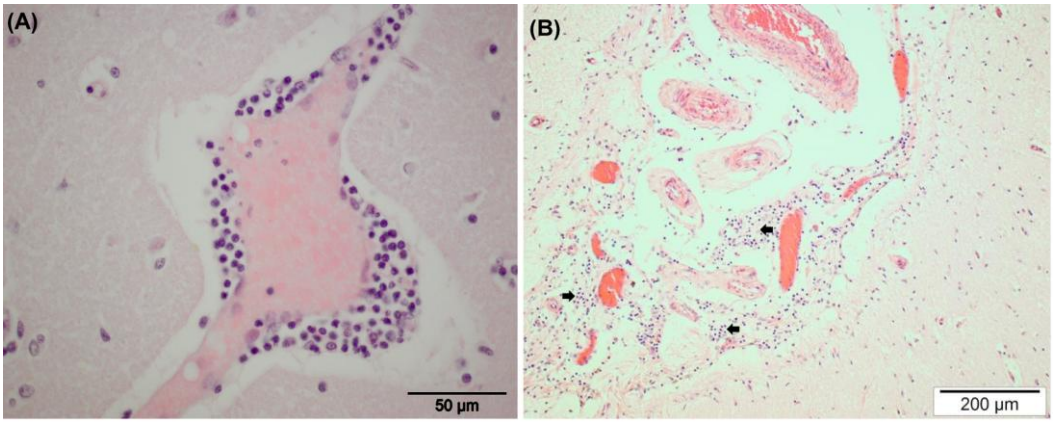


Fig. 1. Histopathology showing (A) Non-suppurative meningoencephalitis represented by mononuclear perivascular cuffs in the gray matter of the cerebral cortex; HE. (B) Meningeal perivascular infiltration of inflammatory cells (arrows); HE.

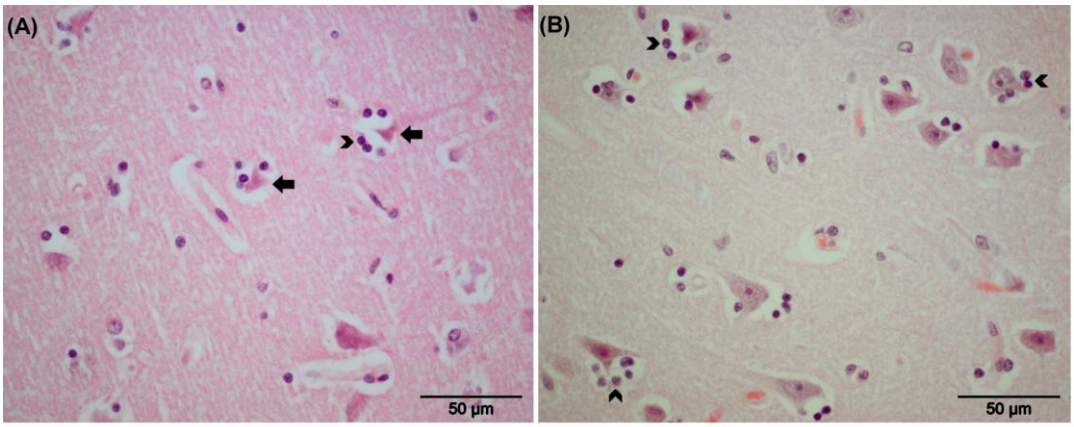


Fig. 2. Histopathology of cerebral cortex showing degenerated neurons (arrows) (A) with mild glial reactions (head of arrows) in (A) and (B); HE.

Table 1. List of positive animals tested in the study and virus infection status.

Animal species	Sample ID	Sex	Age (years)	Observations	Infection
Plains zebra	WP1	f	2	unknown	Lytic EHV-9
Grevy's zebra	CG2	f	23	Found lying in the barn. No clinical signs were observed. Intramuscular bleeding along the femoral shaft was found during necropsy.	Lytic EHV-9
Grevy's zebra	CG3	f	18	Central nervous disorders.	Lytic EHV-9
Plains zebra	CP4	m	24	Signs of ataxia and tremors without improvement after the course of medication.	Lytic EHV-1+ Latent EHV-9
Plains zebra	CP5	m	2	Cardiomyopathy was noticed during necropsy.	Latent zebra-borne EHV-1
Grevy's zebra	CG6	m	11	Massive intra-abdominal hemorrhage followed by hypovolemic shock as a result of surgical complications.	Latent EHV-9

3.3. Detection of latent infection

To test for latency, DNA and RNA were extracted from respiratory lymph nodes and trigeminal ganglia. The RT-qPCR was applied to target the late *gB* gene using DNA samples that were extracted from lymph nodes and trigeminal ganglia as a template. Out of 10 animals

tested, three DNA samples obtained from trigeminal ganglia of two captive plains (CP4, CP5) and one Grevy's (CG6) zebras tested positive for the presence of the *gB* gene. The *gB* DNA load ranged from 8 to 65.3 gene copies per million cells (Table 2). Since the identification of the virus species and individual strains using the short qPCR product is equivocal, partial coding sequences of both *gB* and *Pol* were amplified by conventional PCR. The results showed that the two zebras (CP4 and CG6) and the third zebra (CP5) were positive for EHV-9 and zebra-borne EHV-1, respectively. CG2 and CG3 tested negative for the ganglia (representing lytic infections) and thus could not be included in the latency analysis. CP4, in contrast, had both a lytic (EHV-1) and latent (EHV-9) infection and thus was included in both analyses.

To determine whether the viral DNA detected in the trigeminal ganglia was in a latent state, cDNA was tested for the presence of mRNA transcripts for *gB*. Only RNA extractions that tested negative for genomic DNA contamination using β-glucuronidase control PCRs had been transcribed to cDNA and used. In the latent state, virus is represented by the presence of the viral genome (*gB* DNA), absence of active *gB* expression (represented by absence of mRNA), and the active expression of LATs (Pusterla et al., 2009). EHV-1 and EHV-9 *gB* DNA was detected in all positive samples (CP4, CG6, and CP5), with an absence of active *gB* transcripts (Table 2). Expression of LATs was determined by using a specific TaqMan probe. All positive samples (CP4, CG6, and CP5) displayed signals for transcriptional activity of LATs (Table 2). All positive and negative control samples gave the expected results. These findings indicate that the EHV DNA detected in the trigeminal ganglia was in the latent state. Latent herpesvirus DNA was not detected in any of the tested lymph nodes. Interestingly, these results together with the PCR results showed that zebra (CP4) was co-infected with both EHV-1 (lytic) and EHV-9 (latent) virus.

3.4. Genetic diversity and phylogenetic analyses

The mean nucleotide diversity (π) estimated for EHV-1 and EHV-9 based on *gB* sequences was 0.008% (SE ±0.004) and 0.004% (SE ±0.002), respectively. The π value based on *Pol* sequences was estimated at 0.007% (SE ±0.002) and 0.001% for EHV-1 and EHV-9, respectively. These results indicate lower genetic diversity among EHV-9 sequences compared with EHV-1.

The phylogenetic relationships of the detected EHV-1 and EHV-9, of lytic and latent infections, were inferred from the sequences of the *Pol* and *gB* genes (Fig. 3) as well as the *UL49.5* gene (Supplementary Fig. 2). Virus sequences from lytic infection in WP1 CG2, and CG3, plus the latent infections in CP4 and CG6 latent infections, exhibited 98–99% similarity

to EHV-9 reference sequences. The *gB* sequence of the detected EHV-9 either in captive or wild zebras clustered with a giraffe EHV-9 isolate, forming a sister clade to gazelle EHV-9 isolates (Fig. 3A). The phylogenetic tree inferred from the *Pol* sequences showed that the CG2 lytic, WP1 lytic, CG3 lytic, CP4 latent, and CG6 latent isolates clustered with EHV-9 sequences from gazelle and giraffe (Fig. 3B).

The tree inferred from the *UL49.5* sequences confirmed that the detected isolates belong to the EHV-9 clade (Supplementary Fig. 2). CP4 latent sequences were not included in the phylogenetic analysis of *UL49.5* due to insufficient DNA. Phylogenetic analyses of EHV-9 viruses circulating in zoos demonstrated minimal differences among sequences from captive animals, with only small divergences from the wild strain. In contrast, the partial coding sequences of the three genes determined from the lytic infection of CP4 clustered with horse EHV-1 sequences. CP5 sequences (latent infection) was placed in a group that includes EHV-1 from gazelle, polar bear, onager, Indian rhinoceros, and zebra, and is more distantly related to horse-derived EHV-1 sequences (Fig. 3 and Supplementary Fig. 2).

Table 2. Latent viral DNA load tested by RT-qPCR and performed on DNA and cDNA obtained from trigeminal ganglia of three zebras.

Samples ID	Viral load gDNA gB (copies/million cells)	Transcriptional activity of *gB* (mRNA)	Transcriptional activity of LATs (copies/million cells)	Virus strain
CP4	8	Negative	107	EHV-9
CP5	65.3	Negative	124	EHV-1
CG6	33.3	Negative	174	EHV-9

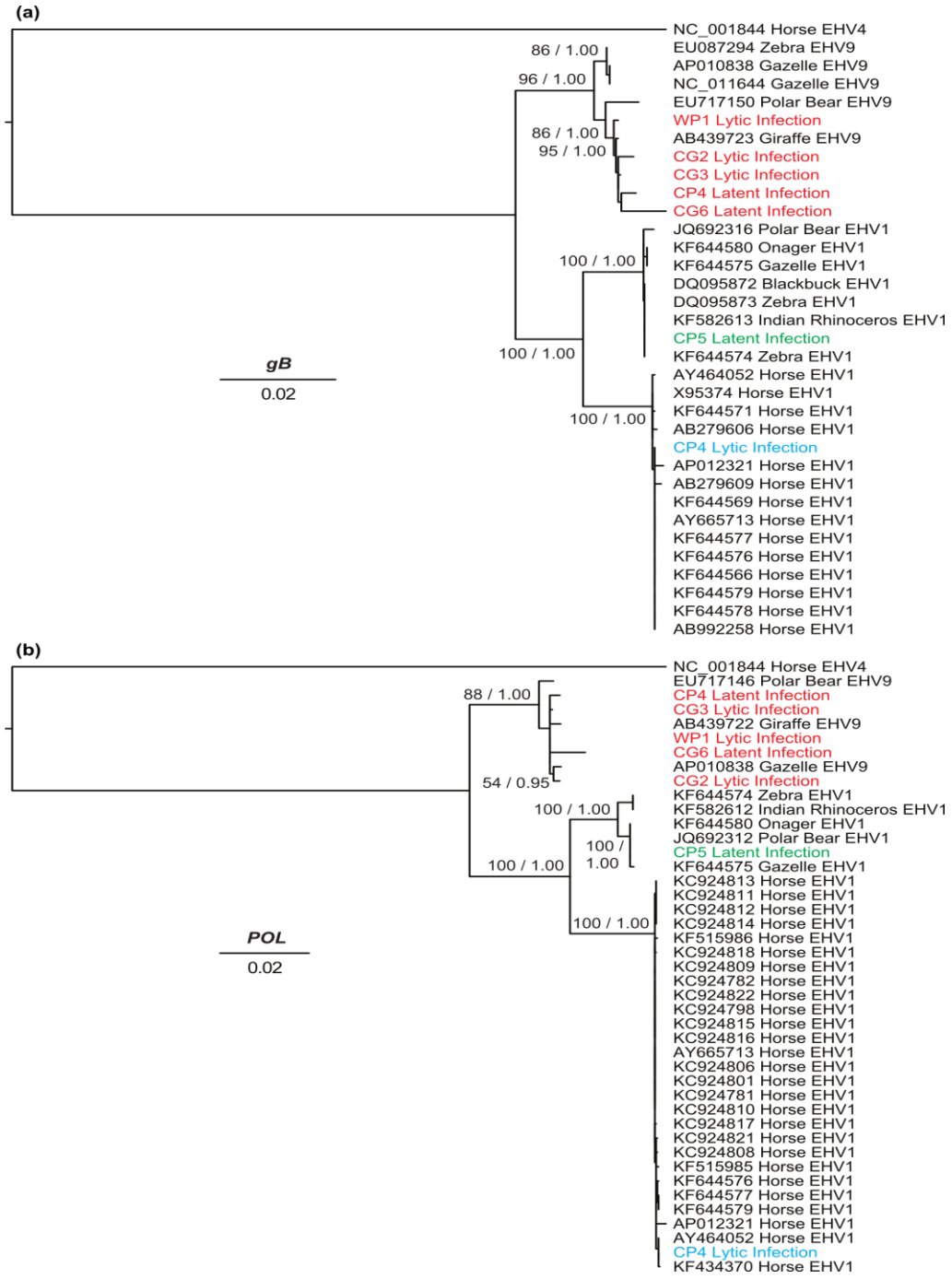


Fig. 3. Phylogenetic trees inferred using maximum likelihood from nucleotide sequences of (A) *gB* and (B) *Pol* genes for the six zebras WP1, CG2, CG3, CP4, CP5, and CG6 and other equine herpesviruses. Reference sequences are indicated by GenBank accession number, species from which the sequence was isolated, and viral strain. The novel EHV-9 sequences are in red, the novel EHV-1-horse like zebra sequence is in blue, and the novel zebra-EHV-1 sequence is in green. The trees are shown with branches lengths scaled to nucleotide substitutions per site. Selected nodes are labeled with maximum-likelihood bootstrap support values and posterior probabilities, separated by a slash "/".

4. Discussion

A key property of alphaherpesviruses is their ability to establish life-long latency in natural hosts where they can evade the host immune system (Ma et al., 2013). This makes elimination of herpesviruses almost impossible and helps them maintain long-term infections in their hosts. Upon stimulation by various cues, the virus can reactivate and be transmitted to susceptible hosts, which may occur with or without overt clinical disease. Uncontrolled reactivation can have a strong impact on zoos, where different susceptible animal species (including endangered animals) are kept together and can result in fatal cross-species transmissions. There have been no previous reports of EHV-1 or EHV-9 latency in zoo animals, except for EHV-1 in horses. Only one study described the possible existence of latent EHV-9 infection in a wild zebra (Borchers et al., 2008). However, the authors did not confirm the actual state of latency; for example, absent transcriptional activity of the *gB* gene and presence of LATs was not demonstrated. The conclusion of latency was based on the presence of viral *gB* DNA in the tested samples.

In the current study we confirmed latent EHV-1 and EHV-9 infections in zebras by detection of active LAT transcripts with the simultaneous absence of *gB* mRNA in the sensory ganglia of zebras. Recently, EHV-1 and EHV-9 have been shown to spread beyond their natural hosts and to infect other zoo animal species, causing fatal neurological disorders (Abdelgawad et al., 2014; Fukushi et al., 1997; Greenwood et al., 2012; Kasem et al., 2008; Schrenzel et al., 2008; Wohlsein et al., 2011). Latency of EHV-1 and EHV-9 in captive zebras suggests that the threat is determined by reactivation in latent individuals, which is currently not predictable. However, clinically healthy latent animals could reactivate and threaten both natural and unexpected hosts.

None of the three latently infected animals showed clinical signs or lesions related to EHV infection, except for the co-infected animal (CP4) in which EHV-1 was lytically replicating. The animal displayed signs of ataxia and tremors before death. Among the three EHV-9-infected zebras, signs of ataxia followed by nervous manifestations were detected in one zebra. Previous reports only described nervous manifestations associated with EHV-9 infection of unnatural hosts, but not equids (Fukushi et al., 1997; Kasem et al., 2008; Schrenzel et al., 2008). Even after experimental infection of horses with EHV-9, only viremia was detected without evidence of neuronal manifestations (Taniguchi et al., 2000a). The ability of the virus to reach the CNS inducing histopathological changes was detected under both natural and experimental infection of unnatural hosts, with the forebrain predominantly affected (Fukushi et al., 2000; Fukushi et al., 1997; Kodama et al., 2007; Taniguchi et al.,

2000b; Yanai et al., 2003a; Yanai et al., 2003b). In the current study, non-suppurative meningoencephalitis was detected for the first time in the cerebral cortex in a zebra, demonstrating the ability of the virus to cross the blood-brain barrier and to cause pathological changes. Whether EHV-9 reaches the nervous system through the nervous tissue or blood remains to be investigated.

Previous reports have described the contribution of homologous recombination to the evolution of many herpesviruses, including HSV-1, HSV-2, EHV-1, EHV-4 and varicella-zoster virus (VZV) (Bowden et al., 2004; Bowden et al., 2006; Norberg, 2010; Norberg et al., 2006; Norberg et al., 2015; Pagamjav et al., 2005). In general, recombination requires co-infection and simultaneous replication of two or more viral strains in the same cell. However, little is known about the exact mechanism of how recombination occurs and if recombination events happen directly after primary infection, during latency, or during reactivation (Norberg et al., 2015). Recently, a recombinant EHV-1 containing EHV-9 sequences in the *Pol* gene was detected in a polar bear and Indian rhinoceros, both of which died after nervous manifestations in Germany (Abdelgawad et al., 2014; Greenwood et al., 2012). It was postulated that zebra might be the host where this recombination took place (Greenwood et al., 2012). We demonstrated the co-occurrence of EHV-1 and EHV-9, with high prevalence, among zebras through detection of neutralizing antibodies of both viruses using a discriminatory ELISA assay (Abdelgawad et al., 2015). In the current study, we have been able to confirm this result further with the detection of co-infection with both EHV-1 and EHV-9 in a captive plains zebra (CP4). Taken together, the results support the notion that the zebra serves as a natural host for both viruses, potentially allowing recombination to take place. In turn, this could lead to the generation of viral strains with altered host specificity and virulence.

In contrast with apparently common variation generated by recombination among EHVs, little sequence variation was observed for EHV-9 in the current study. This result suggests that the evolution of this virus is slow or that natural selection constrains diversification. However, we observed two clades, one grouping gazelle and zebra sequences, and another grouping the polar bear, giraffe and all of the zebra EHV-9 sequences identified in the current study. EHV-1 demonstrated higher sequence diversity, with a clade predominantly grouping domestic horses and a mixed species clade containing the recombinant polar bear, Asian rhinoceros, onager and zebra sequences. Surprisingly, the EHV-1 sequence of the captive zebra (CP4) exhibiting latent EHV-9 infection and lytic EHV-1 infection was most closely related to horse EHV-1. Whether this indicates that the

zebra was infected by domestic horses in captivity, or whether zebras carry this strain naturally, remains to be confirmed in further epidemiological studies.

Virus diversification, co-infection and subsequent recombination have the potential to influence the pathogenicity, virulence and host range of different viruses. All of these variables may increase the severity and outcome of disease episodes, which have to take into account the growing list of the alphaherpesviruses detected recently in different animal species. Latency and reactivation constitute an important epidemiological advantage for the virus, given that sporadic cases of abortion and neurological disease can occur without an external source of infection. This in particular may have pathogenic consequences in zebras and subsequently in other animals, leading to further fatal cross-species transmissions.

5. Conclusions

In conclusion, we propose that EHV-9 infection is circulating between zoo and wild zebras, inducing pathological changes. The ability of EHV-1 and EHV-9 to induce lytic infection followed by latent infection in zebra secures the virus infection and allows it to keep circulating between different animal species. Additionally, the virus co-infection in zebra may raise the chance of virus recombination, with the potential to cause severe losses in zoos where different animal species are kept together.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

We thank Benjamin Lamglait, Réserve Africaine de Sigean (France), Gudrun Wibbelt (Leibniz Institute for Zoo and Wildlife Research (Germany), and Julia Gräger (Zoo Hannover, Germany) for providing us with the samples. We also thank Ahmed Kheimar (Freie Universität Berlin) for his supportive discussion and the Tanzania Wildlife Research Institute and Tanzanian National Parks Authority for their support. AA was supported by an Else Neumann scholarship provided through the Nachwuchsförderungsgesetz des Landes Berlin (NaFöG). MLE, NO and ADG were supported by a grant from the Leibniz Gemeinschaft, SAW-2015-IZW-1 440. The research described in this article was approved by the Internal Ethics Committee of the Leibniz-Institute for Zoo and Wildlife Research (IZW), Approval no. 2013-05-03.

References

Abdelgawad, A., Azab, W., Damiani, A.M., Baumgartner, K., Will, H., Osterrieder, N., Greenwood, A.D., 2014. Zebra-borne equine herpesvirus type 1 (EHV-1) infection in non-African captive mammals. Vet Microbiol 169, 102-106.

Abdelgawad, A., Hermes, R., Damiani, A., Lamglait, B., Czirjak, G.A., East, M., Aschenborn, O., Wenker, C., Kasem, S., Osterrieder, N., Greenwood, A.D., 2015. Comprehensive Serology Based on a Peptide ELISA to Assess the Prevalence of Closely Related Equine Herpesviruses in Zoo and Wild Animals. PloS one 10, e0138370.

Aleman, M., Pickles, K.J., Simonek, G., Madigan, J.E., 2012. Latent equine herpesvirus-1 in trigeminal ganglia and equine idiopathic headshaking. Journal of veterinary internal medicine / American College of Veterinary Internal Medicine 26, 192-194.

Allen, G.P., 2004. Equid herpesvirus- 1 (EHV-1) and -4 (EHV-4) infections. IN Infectious Diseases of Livestock, (Ed.) JAW Coetzer and RC Tustin. Oxford Press (Cape Town), Chapter 76, pp 829-859.

Baxi, M.K., Efstathiou, S., Lawrence, G., Whalley, J.M., Slater, J.D., Field, H.J., 1995. The detection of latency-associated transcripts of equine herpesvirus 1 in ganglionic neurons. The Journal of general virology 76 (Pt 12), 3113-3118.

Borchers, K., Frolich, K., 1997. Antibodies against equine herpesviruses in free-ranging mountain zebras from Namibia. Journal of wildlife diseases 33, 812-817.

Borchers, K., Lieckfeldt, D., Ludwig, A., Fukushi, H., Allen, G., Fyumagwa, R., Hoare, R., 2008. Detection of Equid herpesvirus 9 DNA in the trigeminal ganglia of a Burchell's zebra from the Serengeti ecosystem. The Journal of veterinary medical science / the Japanese Society of Veterinary Science 70, 1377-1381.

Bowden, R., Sakaoka, H., Donnelly, P., Ward, R., 2004. High recombination rate in herpes simplex virus type 1 natural populations suggests significant co-infection. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases 4, 115-123.

Bowden, R., Sakaoka, H., Ward, R., Donnelly, P., 2006. Patterns of Eurasian HSV-1 molecular diversity and inferences of human migrations. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases 6, 63-74.

D'Haene, B., Vandesompele, J., Hellemans, J., 2010. Accurate and objective copy number profiling using real-time quantitative PCR. Methods 50, 262-270.

Davison, A.J., Eberle, R., Ehlers, B., Hayward, G.S., McGeoch, D.J., Minson, A.C., Pellett, P.E., Roizman, B., Studdert, M.J., Thiry, E., 2009. The order Herpesvirales. Arch Virol 154, 171-177.

Dunowska, M., 2014. A review of equid herpesvirus 1 for the veterinary practitioner. Part B: pathogenesis and epidemiology. N Z Vet J. 62(4):179-88. doi: 10.1080/00480169.2014.899946.

Escalera-Zamudio, M., Zepeda-Mendoza, M.L., Loza-Rubio, E., Rojas-Anaya, E., Mendez-Ojeda, M.L., Arias, C.F., Greenwood, A.D., 2015. The evolution of bat nucleic acid-sensing Toll-like receptors. Molecular ecology 24, 5899-5909.

Fukushi, H., Taniguchi, A., Yasuda, K., Yanai, T., Masegi, T., Yamaguchi, T., Hirai, K., 2000. A hamster model of equine herpesvirus 9 induced encephalitis. J Neurovirol 6, 314-319.

Fukushi, H., Tomita, T., Taniguchi, A., Ochiai, Y., Kirisawa, R., Matsumura, T., Yanai, T., Masegi, T., Yamaguchi, T., Hirai, K., 1997. Gazelle herpesvirus 1: a new neurotropic herpesvirus immunologically related to equine herpesvirus 1. Virology 227, 34-44.

Fukushi, H., Yamaguchi, T., Yamada, S., 2012. Complete genome sequence of equine herpesvirus type 9. J Virol 86, 13822.

Goodman, L.B., Loregian, A., Perkins, G.A., Nugent, J., Buckles, E.L., Mercorelli, B., Kydd, J.H., Palu, G., Smith, K.C., Osterrieder, N., Davis-Poynter, N., 2007. A point mutation in a herpesvirus polymerase determines neuropathogenicity. PLoS pathogens 3, e160.

GP, A., 2004. Allen GP, JH Kydd, JD Slater and KC Smith. Equid herpesvirus- 1 (EHV-1) and -4 (EHV-4) infections. IN Infectious Diseases of Livestock, (Ed.) JAW Coetzer and RC Tustin. Oxford Press (Cape Town), Chapter 76, pp 829-859, 2004.

Greenwood, A.D., Tsangaras, K., Ho, S.Y., Szentiks, C.A., Nikolin, V.M., Ma, G., Damiani, A., East, M.L., Lawrenz, A., Hofer, H., Osterrieder, N., 2012. A potentially fatal mix of herpes in zoos. Curr Biol 22, 1727-1731.

Guo, X., Izume, S., Okada, A., Ohya, K., Kimura, T., Fukushi, H., 2014. Full genome sequences of zebra-borne equine herpesvirus type 1 isolated from zebra, onager and Thomson's gazelle. The Journal of veterinary medical science / the Japanese Society of Veterinary Science 76, 1309-1312.

Kasem, S., Yamada, S., Kiupel, M., Woodruff, M., Ohya, K., Fukushi, H., 2008. Equine herpesvirus type 9 in giraffe with encephalitis. Emerg Infect Dis 14, 1948-1949.

Klieforth, R., Maalouf, G., Stalis, I., Terio, K., Janssen, D., Schrenzel, M., 2002. Malignant catarrhal fever-like disease in Barbary red deer (Cervus elaphus barbarus) naturally infected with a virus resembling alcelaphine herpesvirus 2. Journal of clinical microbiology 40, 3381-3390.

Kodama, A., Yanai, T., Yomemaru, K., Sakai, H., Masegi, T., Yamada, S., Fukushi, H., Kuraishi, T., Hattori, S., Kai, C., 2007. Acute neuropathogenicity with experimental infection of equine herpesvirus 9 in common marmosets (Callithrix jacchus). J Med Primatol 36, 335-342.

Ma, G., Azab, W., Osterrieder, N., 2013. Equine herpesviruses type 1 (EHV-1) and 4 (EHV-4)--masters of co-evolution and a constant threat to equids and beyond. Vet Microbiol 167, 123-134.

Nei, M., Jin, L., 1989. Variances of the average numbers of nucleotide substitutions within and between populations. Molecular biology and evolution 6, 290-300.

Norberg, P., 2010. Divergence and genotyping of human alpha-herpesviruses: an overview. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases 10, 14-25.

Norberg, P., Bergstrom, T., Liljeqvist, J.A., 2006. Genotyping of clinical herpes simplex virus type 1 isolates by use of restriction enzymes. Journal of clinical microbiology 44, 4511-4514.

Norberg, P., Bergstrom, T., Rekabdar, E., Lindh, M., Liljeqvist, J.A., 2004. Phylogenetic analysis of clinical herpes simplex virus type 1 isolates identified three genetic groups and recombinant viruses. J Virol 78, 10755-10764.

Norberg, P., Depledge, D.P., Kundu, S., Atkinson, C., Brown, J., Haque, T., Hussaini, Y., MacMahon, E., Molyneaux, P., Papaevangelou, V., Sengupta, N., Koay, E.S., Tang, J.W., Underhill, G.S., Grahn, A., Studahl, M., Breuer, J., Bergstrom, T., 2015. Recombination of Globally Circulating Varicella-Zoster Virus. J Virol 89, 7133-7146.

Pagamjav, O., Sakata, T., Matsumura, T., Yamaguchi, T., Fukushi, H., 2005. Natural recombinant between equine herpesviruses 1 and 4 in the ICP4 gene. Microbiology and immunology 49, 167-179.

Pandey, S.N., Khawaja, H., Chen, Y.W., 2015. Culture Conditions Affect Expression of DUX4 in FSHD Myoblasts. Molecules 20, 8304-8315.

Pusterla, N., Wilson, W.D., Mapes, S., Finno, C., Isbell, D., Arthur, R.M., Ferraro, G.L., 2009. Characterization of viral loads, strain and state of equine herpesvirus-1 using real-time PCR in horses following natural exposure at a racetrack in California. Veterinary journal 179, 230-239.

Ramakrishnan, R., Poliani, P.L., Levine, M., Glorioso, J.C., Fink, D.J., 1996. Detection of herpes simplex virus type 1 latency-associated transcript expression in trigeminal ganglia by in situ reverse transcriptase PCR. J Virol 70, 6519-6523.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Hohna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic biology 61, 539-542.

Schrenzel, M.D., Tucker, T.A., Donovan, T.A., Busch, M.D., Wise, A.G., Maes, R.K., Kiupel, M., 2008. New hosts for equine herpesvirus 9. Emerg Infect Dis 14, 1616-1619.

Smith, D.J., Iqbal, J., Purewal, A., Hamblin, A.S., Edington, N., 1998. In vitro reactivation of latent equid herpesvirus-1 from CD5+/CD8+ leukocytes indirectly by IL-2 or chorionic gonadotrophin. The Journal of general virology 79 (Pt 12), 2997-3004.

Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30, 1312-1313.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular biology and evolution 24, 1596-1599.

Taniguchi, A., Fukushi, H., Matsumura, T., Yanai, T., Masegi, T., Hirai, K., 2000a. Pathogenicity of a new neurotropic equine herpesvirus 9 (gazelle herpesvirus 1) in horses. The Journal of veterinary medical science / the Japanese Society of Veterinary Science 62, 215-218.

Taniguchi, A., Fukushi, H., Yanai, T., Masegi, T., Yamaguchi, T., Hirai, K., 2000b. Equine herpesvirus 9 induced lethal encephalomyelitis in experimentally infected goats. Arch Virol 145, 2619-2627.

Telford, E.A., Watson, M.S., Perry, J., Cullinane, A.A., Davison, A.J., 1998. The DNA sequence of equine herpesvirus-4. The Journal of general virology 79 (Pt 5), 1197-1203.

Thiry, E., Meurens, F., Muylkens, B., McVoy, M., Gogev, S., Thiry, J., Vanderplasschen, A., Epstein, A., Keil, G., Schynts, F., 2005. Recombination in alphaherpesviruses. Reviews in medical virology 15, 89-103.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic acids research 22, 4673-4680.

VanDevanter, D.R., Warrener, P., Bennett, L., Schultz, E.R., Coulter, S., Garber, R.L., Rose, T.M., 1996. Detection and analysis of diverse herpesviral species by consensus primer PCR. Journal of clinical microbiology 34, 1666-1671.

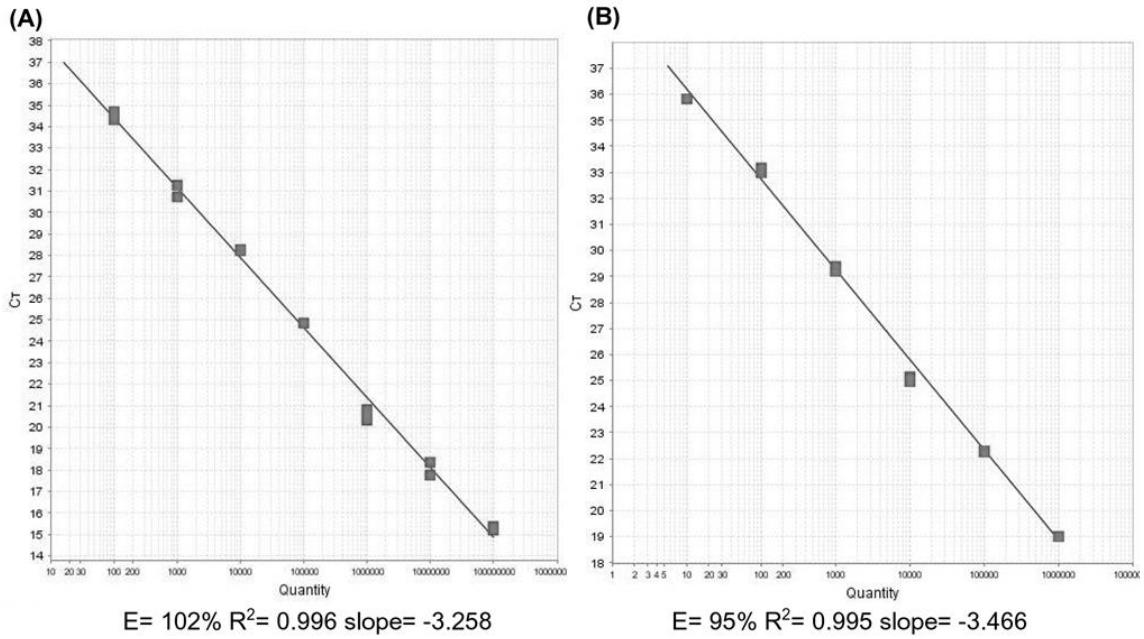
Welch, H.M., Bridges, C.G., Lyon, A.M., Griffiths, L., Edington, N., 1992. Latent equid herpesviruses 1 and 4: detection and distinction using the polymerase chain reaction and co-cultivation from lymphoid tissues. The Journal of general virology 73 (Pt 2), 261-268.

Wohlsein, P., Lehmbecker, A., Spitzbarth, I., Algermissen, D., Baumgartner, W., Boer, M., Kummrow, M., Haas, L., Grummer, B., 2011. Fatal epizootic equine herpesvirus 1 infections in new and unnatural hosts. Vet Microbiol 149, 456-460.

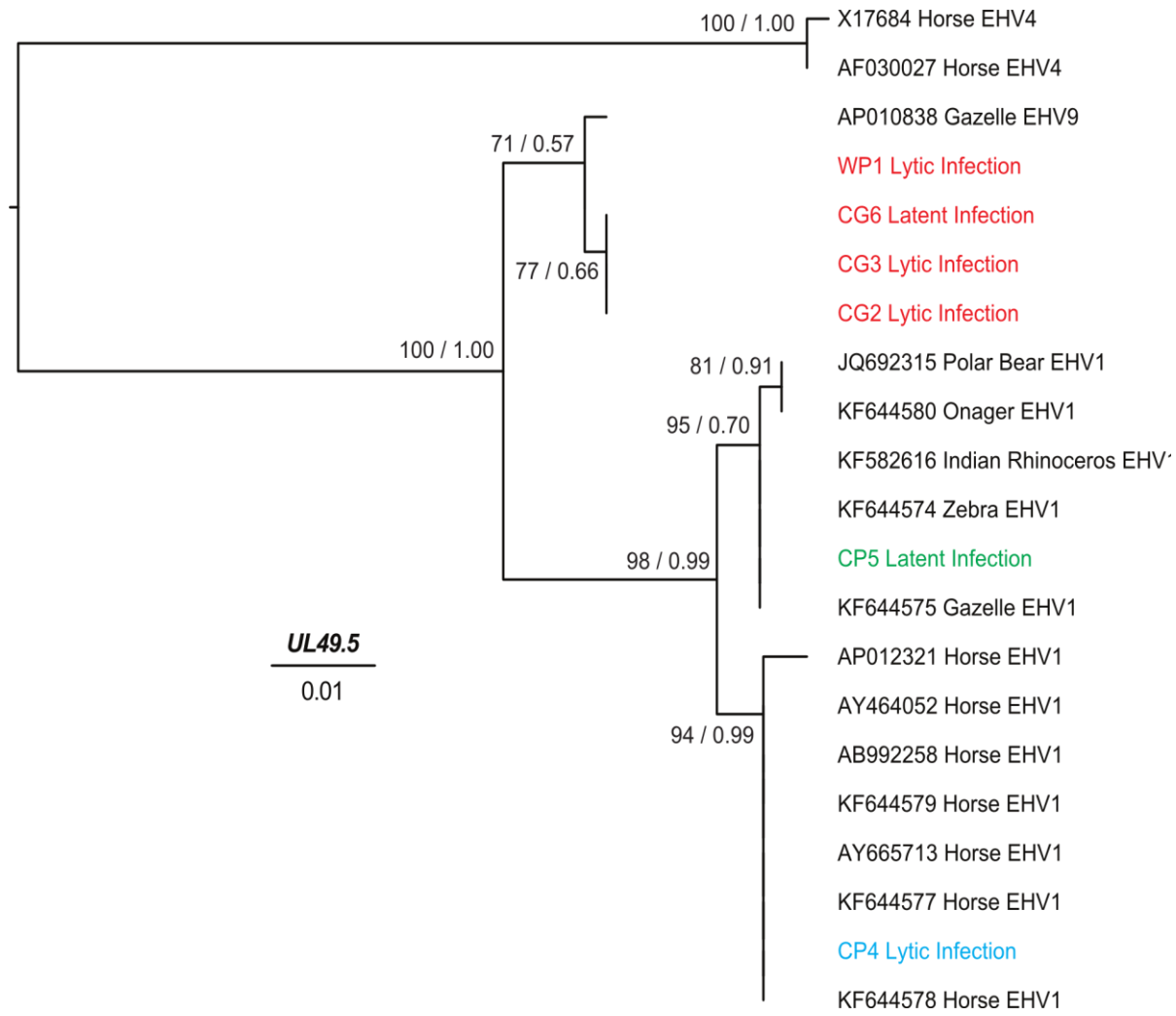
Yanai, T., Fujishima, N., Fukushi, H., Hirata, A., Sakai, H., Masegi, T., 2003a. Experimental infection of equine herpesvirus 9 in dogs. Veterinary pathology 40, 263-267.

Yanai, T., Tujioka, S., Sakai, H., Fukushi, H., Hirai, K., Masegi, T., 2003b. Experimental infection with equine herpesvirus 9 (EHV-9) in cats. J Comp Pathol 128, 113-118.

Supplementary material



Supplementary Fig. 1 The RT-qPCR standardizations. The standard curve of 10-fold serial dilutions of (a) *gB* and (b) *ORF63* are shown with a correlation coefficient (R2) of 0.996 and 0.995, respectively.



Supplementary Fig. 2 Phylogenetic tree inferred using maximum likelihood from nucleotide sequences of *UL49.5* gene for the six zebras WP1, CG2, CG3, CP4, CP5, and CG6 and other equine herpesviruses. The novel EHV-9 sequences are in red, the novel EHV-1-horse like zebra sequence is in blue, and the novel zebra-EHV-1 sequence is in green. Selected nodes are labeled with maximum-likelihood bootstrap support values and posterior probabilities, separated by a slash "/".

Supplementary Table 1. List of equid animals analyzed in the study.

Common name	Species/subspecies	Origin	Number
Plains zebra	*Equus quagga*	Wild; WP	7*
Plains zebra	*Equus quagga boehmi*	Captive; CP	3; 1*
Grevy's zebra	*Equus grevyi*	Captive; CG	3
Hartmann's mountain zebra	*Equus zebra hartmannae*	Captive	2
Somali wild ass	*Equus africanus somalicus*	Captive	6; 3*
Donkey	*Equus africanus asinus*	Captive	3*

* Ganglia and lymph nodes were not collected from these animals.

Supplementary Table 2. List of new primers and probes used in the study.

Primer	Sequence
Pol	
AZ11 (F)	5′-AATGTGCGATCTCAGCTTTG-3′
AZ14 (R)	5′-GATCTTTTGTTGTACGACGA-3′
AZ1 (F)	5′-TACAACAAAAGATCTACCAG-3′
AZ2 (R)	5′-GATAGCCAAAGCCACGCCTT-3′
AZ12 (F)	5′-CGTGGCTTTGGCTATCCATA-3′
AZ15 (R)	5′-ATCTCCTGTCTGCTGTACTC-3′
AZ9 (F)	5′-AGGTCCTCTTGGTTAGTTGC-3′
AZ17 (R)	5′-TTAAATTTACACAGACATG-3′
gB	
gB1 (F)	5′-CCATGTCAACGCACTCCC-3′
gB1 (R)	5′-ACAATATCACCGGTGGACAG-3′
gB2 (F)	5′-CTGTCCACCGGTGATATTGT-3′
gB2b (R)	5′-GGTACGGACAGGAGAGACCT-3′
gB (F)	5'-CTTGTGAGATCTAACCGCAC-3′
gB (R)	5′-GGGTATAGAGCTTTCATGGGG-3′
gB3 (F)	5′-AGATATGTAATGCAGATCCG-3′
gB3b (R)	5′-AAATATGAGGTCACACTTT-3′
ORF69–ORF74	
US3 (F)	5′-GACCACCTAACCGACTGGTT-3′
US3 (R)	5′-CGCGTGTAGGGCTTGCGCTC-3′
US4 (F)	5′-CTACCCCTGCTTTCAACGCG-3′
US4 (R)	5′-TGTGTGACTCCCACGAGTGA-3′
US5 (F)	5′-CTTACCCAAATACGCTGAGG-3′
US6 (R)	5′-TCTCGTATGTTGACGAGCCCA-3′
US6 (F)	5′-GCCGCTACAACCACAGCTGT-3′
US7 (R)	5′-AAGCGAAGTTGGAAGTTGAG-3′
US8 (F)	5′-TTAGTGGCTGCGACCACGCT-3′
US8 (R)	5′-ATCCGGAGGCACGGGTCTTG-3′
US9 (F)	5′-CCGGATAACCACCCTGGATT-3′
US10 (R)	5′-CCCCACGCATCGAGTACTGT-3′
US11 (F)	5′-AGTCCAACAAGTTGAACTTT-3′
US11 (R)	5′-TTCATAAAGTGATTTGCGGT-3′
LAT-specific primer	5′-CTGGCTGGTCGAAAGGCTCG-3′
qPCR	
ORF63_LAT (F)	5′-GTGTCTTCGTGAAACATCGG-3′
ORF63_LAT (R)	5′-TGCGGAACATTGTTATGGAT-3′
ORF63_LAT (probe)	5′FAM-TCCTCGTTACAGCCATGCTCGC-TAMRA3′
ORF63 (oligo)	GATGAGATCCGTGACAAAGGGCACAGTGTCTTCGTGAAACAT CGGCCAAAACTGGCGAGTGAGCTCTTCCTCGTTACAGCCATGC TCGCACAGTGTATCCATAACAATGTTCCGCATCAC
B2M (F)	5′-ATG GAA AGC CAA ATT TCC TG-3′
B2M (R)	5′-ACC GGT CGA CTT TCA TCT TC-3′
B2M (probe)	5'HEX- TGG GTT CCA TCC GCC TGA GA - BHQ13′
B2M (oligo)	AGAGAATGGAAAGCCAAATTTCCTGAACTGCTATGTCTCTGG GTTCCATCCGCCTGAGATTGAAATTGATTTGCTAAAGAATGGA GAGAAGATGAAAGTCGACCGGTCAGAC

Chapter 4

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

Azza Abdelgawad[a], Walid Azab[b,c], Armando M. Damiani[b], Katrin Baumgartner[d], Hermann Will[d], Nikolaus Osterrieder[b], Alex D. Greenwood[a]

[a]Leibniz-Institute for Zoo and Wildlife Research, Alfred-Kowalke-Strasse 17, 10315 Berlin, Germany
[b]Institut für Virologie, Freie Universität Berlin, Robert-von-Ostertag-Str. 7-13, 14163 Berlin Germany
[c]Department of Virology, Faculty of Veterinary Medicine, Zagazig University, Egypt
[d]Zoo Nuremberg. Am Tiergarten 30. 90480 Nuremberg, Germany

Veterinary Microbiology, Volume 169, Issues 1-2, 21 February 2014, Pages 102-106
DOI: http://dx.doi.org/10.1016/j.vetmic.2013.12.011

Abstract

Equine herpesvirus type 1 (EHV-1) was detected in an Indian rhinoceros (*Rhinoceros unicornis*), which was euthanized because of severe neurological disease. Encephalitis was suspected and EHV-1 DNA was detected in brain, lung, and spleen tissues. The viral *IR6* protein was detected in lung tissues by western blot analysis. Phylogenetic analyses of EHV-1 sequences amplified from various tissues was nearly identical to one recently described that resulted in both non-fatal and fatal encephalitis in polar bears. This represents transmission of EHV-1 to a species that is not naturally sympatric with the natural host of the virus and broadens the host range to Asian non-equid perissodactyls.

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 burchelii)* and Grevy's zebras *(Equu sgrevyi) (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.

Materials and Methods

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-80ºC. 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).

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).

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 a 1:20,000 dilution was used as a secondary antibody. Reactive bands were visualized by enhanced chemoluminescence (Amersham ECL plus, GE healthcare).

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 1,250 generations.

Results

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 saliva sampled from the clinically co-housed unaffected polar bears.

Table 1. Results of qPCR showed by the threshold cycle (Ct) values per 115 ng DNA.

Name	Tissue sample	EHV-1 *gB* qPCR (Ct) values
Purana	Brain	21
Purana	Lung	31
Purana	Spleen	31
Purana	Liver	Negative
Gregor	Saliva	37
Aleut	Saliva	Negative
Vera	Saliva	Negative
H_2O		Negative

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).

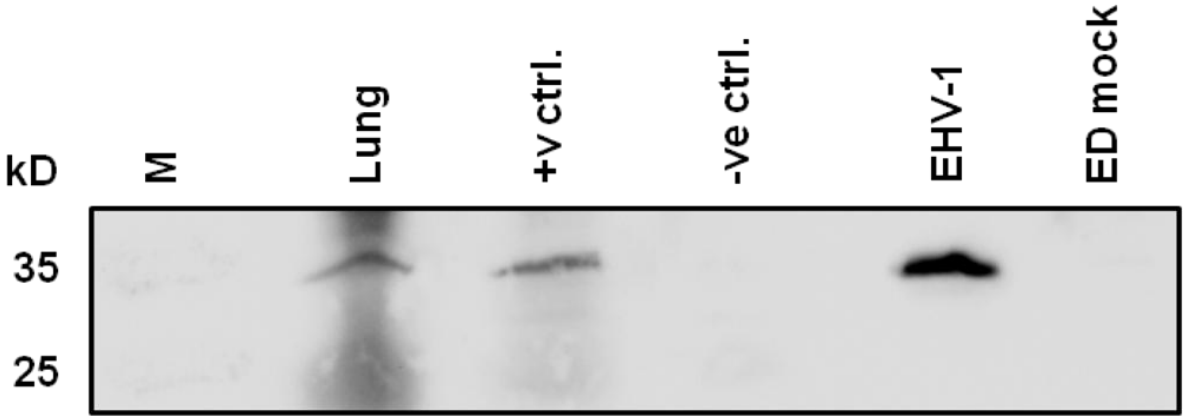


Fig. 1. Detection of equine herpesvirus by Western blot. Cell lysates were prepared and the blots were analyzed by using EHV-1 polyclonal anti-*IR6* antibody. Lysates of EHV-1-infected ED cells and EHV-1-infected horse lung (PC) were included as positive controls. Mock-infected ED cells and rhinoceros lymph node (NC) lysates were included as negative controls. One band of approximately 33 kD was detected in Purana's lung that is not present in mock-infected cells or NC lanes.

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 Baysian 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-KF582616). 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. F3).

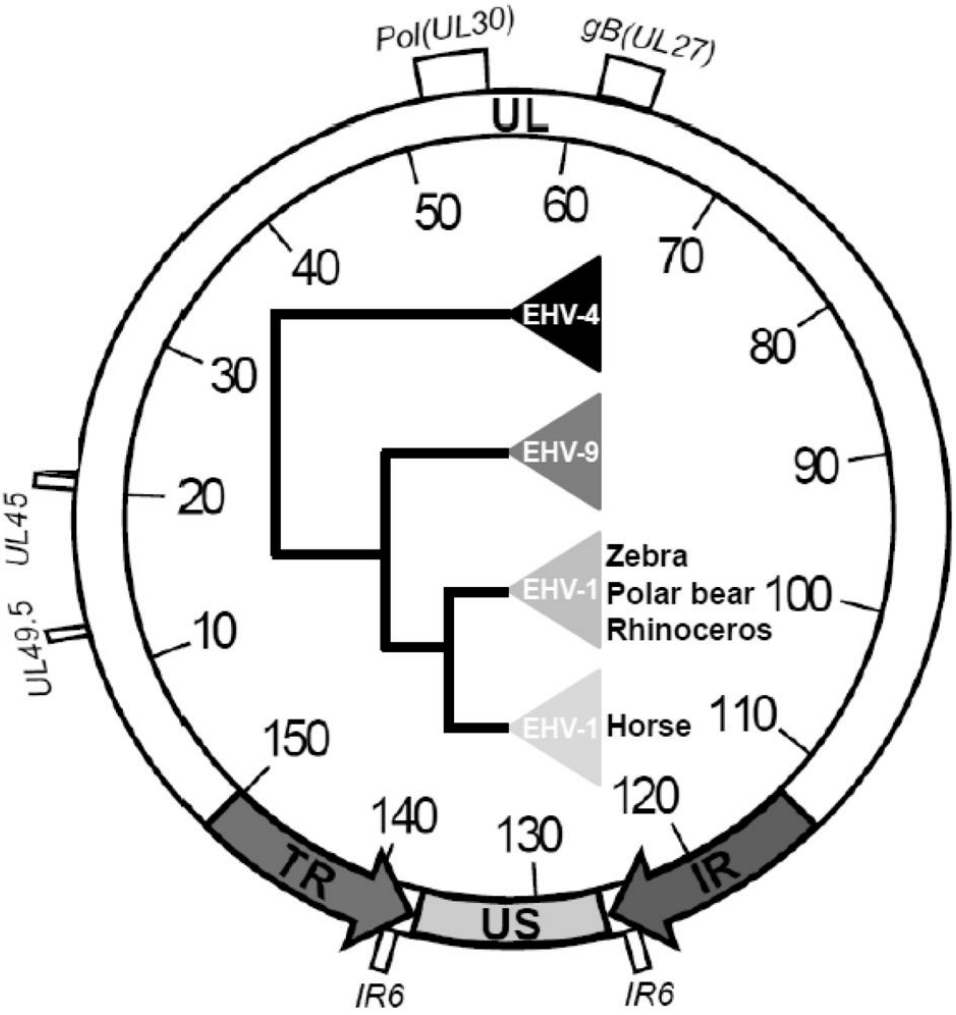


Fig. 2. Phylogeny of EHV sequences detected in an Indian rhino. A summary tree for Maximum Likelihood trees derived from *gB* (UL27), DNA polymerase (*UL30*), *IR6*, *UL45* and *UL49.5* is shown within a depiction of the EHV-1 genome and the relative position of the genes sequenced. Individual trees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

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.

Sequencing 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 meters 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 (*Musmusculus*) are known to rapidly clear EHV-1 infection under experimental conditions (El-Nahass et al., 2011; 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 rhinoceroses 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 not 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.

Acknowledgments

The authors thank Arne Lawrenz for his support during the initial stages of this investigation, Sebastian Bischofberger for technical support, and Kyriakos Tsangaras for assistance with the phylogenetic analyses. AA was supported by an Else Neumann scholarship provided through the Nachwuchsförderungsgesetz des Landes Berlin (NaFöG).

The described research was approved by the Internal Ethics Committee of the Leibniz-institute for Zoo and Wildlife Research (IZW), Approval no. 2012-10-01.

References

Anez, G., Grinev, A., Chancey, C., Ball, C., Akolkar, N., Land, K.J., Winkelman, V., Stramer, S.L., Kramer, L.D., Rios, M., 2013. Evolutionary dynamics of west nile virus in the United States, 1999-2011: phylogeny, selection pressure and evolutionary time-scale analysis. PLoS neglected tropical diseases 7, e2245.

Borchers, K., Bottner, D., Lieckfeldt, D., Ludwig, A., Frolich, K., Klingeborn, B., Widen, F., Allen, G., Ludwig, H., 2006. Characterization of equid herpesvirus 1 (EHV-1) related viruses from captive Grevy's zebra and blackbuck. The Journal of veterinary medical science / the Japanese Society of Veterinary Science 68, 757-760.

Chowdhury, S.I., Ludwig, H., Buhk, H.J., 1988. Molecular biological characterization of equine herpesvirus type 1 (EHV-1) isolates from ruminant hosts. Virus Res 11, 127-139.

Chukeatirote, E., Maharachchikumbura, S.S., Wongkham, S., Sysouphanthong, P., Phookamsak, R., Hyde, K.D., 2012. Cloning and sequence analysis of the cellobiohydrolase I genes from some basidiomycetes. Mycobiology 40, 107-110.

Crandell, R.A., Ichimura, H., Kit, S., 1988. Isolation and comparative restriction endonuclease DNA fingerprinting of equine herpesvirus-1 from cattle. American journal of veterinary research 49, 1807-1813.

El-Nahass, E., El-Habashi, N., Abdelaziz, A.A., Nayel, M., Kasem, S., Fukushi, H., Tuji, H., Hirata, A., Sakai, H., Yanai, T., 2011. Kinetics and Pathogenicity of Oral Infection by Equine Herpesvirus-9 in Mice and Suckling Hamsters. J Comp Pathol.

Fischer-Tenhagen, C., Hamblin, C., Quandt, S., Frolich, K., 2000. Serosurvey for selected infectious disease agents in free-ranging black and white rhinoceros in Africa. Journal of wildlife diseases 36, 316-323.

Fukushi, H., Tomita, T., Taniguchi, A., Ochiai, Y., Kirisawa, R., Matsumura, T., Yanai, T., Masegi, T., Yamaguchi, T., Hirai, K., 1997. Gazelle herpesvirus 1: a new neurotropic herpesvirus immunologically related to equine herpesvirus 1. Virology 227, 34-44.

Ghanem, Y.M., Fukushi, H., Ibrahim, E.S., Ohya, K., Yamaguchi, T., Kennedy, M., 2008. Molecular phylogeny of equine herpesvirus 1 isolates from onager, zebra and Thomson's gazelle. Arch Virol 153, 2297-2302.

Goodman, L.B., Loregian, A., Perkins, G.A., Nugent, J., Buckles, E.L., Mercorelli, B., Kydd, J.H., Palu, G., Smith, K.C., Osterrieder, N., Davis-Poynter, N., 2007. A point mutation in a herpesvirus polymerase determines neuropathogenicity. PLoS pathogens 3, e160.

Greenwood, A.D., Tsangaras, K., Ho, S.Y., Szentiks, C.A., Nikolin, V.M., Ma, G., Damiani, A., East, M.L., Lawrenz, A., Hofer, H., Osterrieder, N., 2012. A potentially fatal mix of herpes in zoos. Curr Biol 22, 1727-1731.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754-755.

Hussey, S.B., Clark, R., Lunn, K.F., Breathnach, C., Soboll, G., Whalley, J.M., Lunn, D.P., 2006. Detection and quantification of equine herpesvirus-1 viremia and nasal shedding by real-time polymerase chain reaction. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 18, 335-342.

Ibrahim, E.S., Kinoh, M., Matsumura, T., Kennedy, M., Allen, G.P., Yamaguchi, T., Fukushi, H., 2007. Genetic relatedness and pathogenicity of equine herpesvirus 1 isolated from onager, zebra and gazelle. Arch Virol 152, 245-255.

Lunn, D.P., Davis-Poynter, N., Flaminio, M.J., Horohov, D.W., Osterrieder, K., Pusterla, N., Townsend, H.G., 2009. Equine herpesvirus-1 consensus statement. Journal of veterinary internal medicine / American College of Veterinary Internal Medicine 23, 450-461.

Ma, G., Azab, W., Osterrieder, N., 2013. Equine herpesviruses type 1 (EHV-1) and 4 (EHV-4)-Masters of co-evolution and a constant threat to equids and beyond. Vet Microbiol.

O'Callaghan, D.J., Colle, C.F., 3rd, Flowers, C.C., Smith, R.H., Benoit, J.N., Bigger, C.A., 1994. Identification and initial characterization of the IR6 protein of equine herpesvirus 1. J Virol 68, 5351-5364.

Osterrieder, N., Holden, V.R., Brandmuller, C., Neubauer, A., Kaaden, O.R., O'Callaghan, D.J., 1996. The equine herpesvirus 1 IR6 protein is nonessential for virus growth in vitro and modified by serial virus passage in cell culture. Virology 217, 442-451.

Posada, D., 2008. jModelTest: phylogenetic model averaging. Molecular biology and evolution 25, 1253-1256.

Rebhun, W.C., Jenkins, D.H., Riis, R.C., Dill, S.G., Dubovi, E.J., Torres, A., 1988. An epizootic of blindness and encephalitis associated with a herpesvirus indistinguishable from equine herpesvirus I in a herd of alpacas and llamas. J Am Vet Med Assoc 192, 953-956.

Schrenzel, M.D., Tucker, T.A., Donovan, T.A., Busch, M.D., Wise, A.G., Maes, R.K., Kiupel, M., 2008. New hosts for equine herpesvirus 9. Emerg Infect Dis 14, 1616-1619.

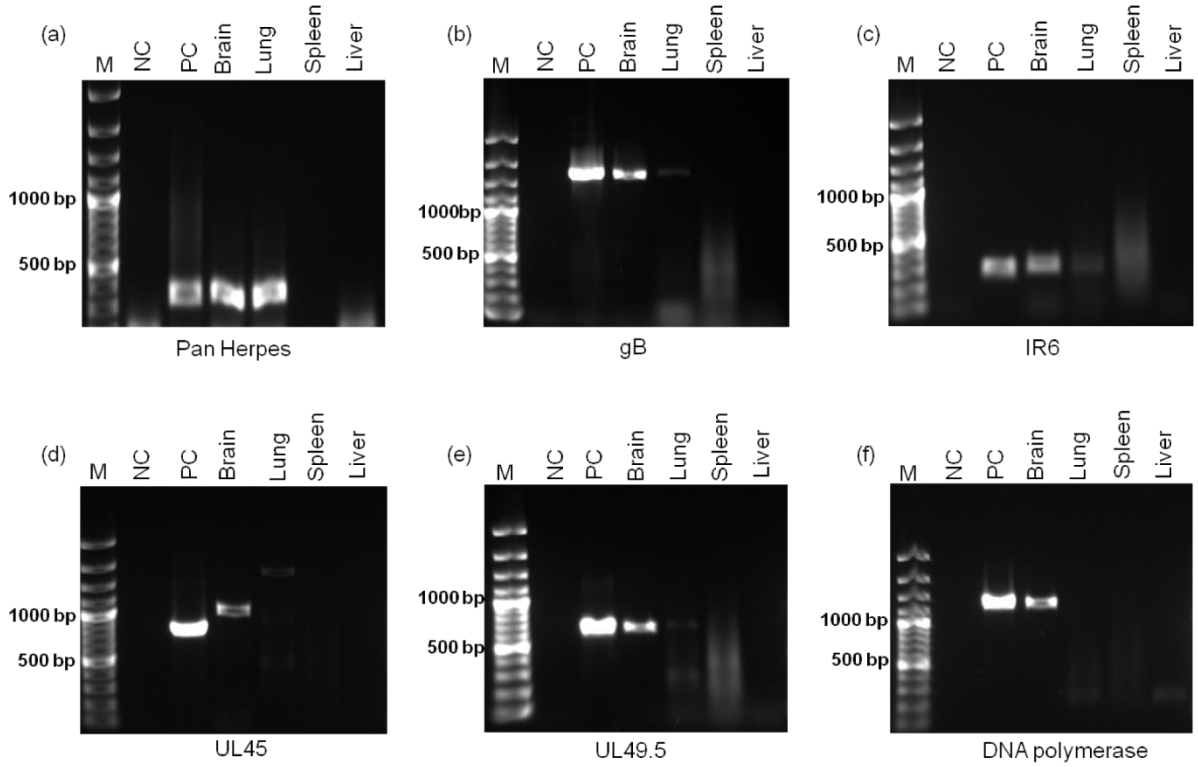
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic acids research 22, 4673-4680.

VanDevanter, D.R., Warrener, P., Bennett, L., Schultz, E.R., Coulter, S., Garber, R.L., Rose, T.M., 1996. Detection and analysis of diverse herpesviral species by consensus primer PCR. Journal of clinical microbiology 34, 1666-1671.

von Einem, J., Smith, P.M., Van de Walle, G.R., O'Callaghan, D.J., Osterrieder, N., 2007. In vitro and in vivo characterization of equine herpesvirus type 1 (EHV-1) mutants devoid of the viral chemokine-binding glycoprotein G (gG). Virology 362, 151-162.

Supplementary data

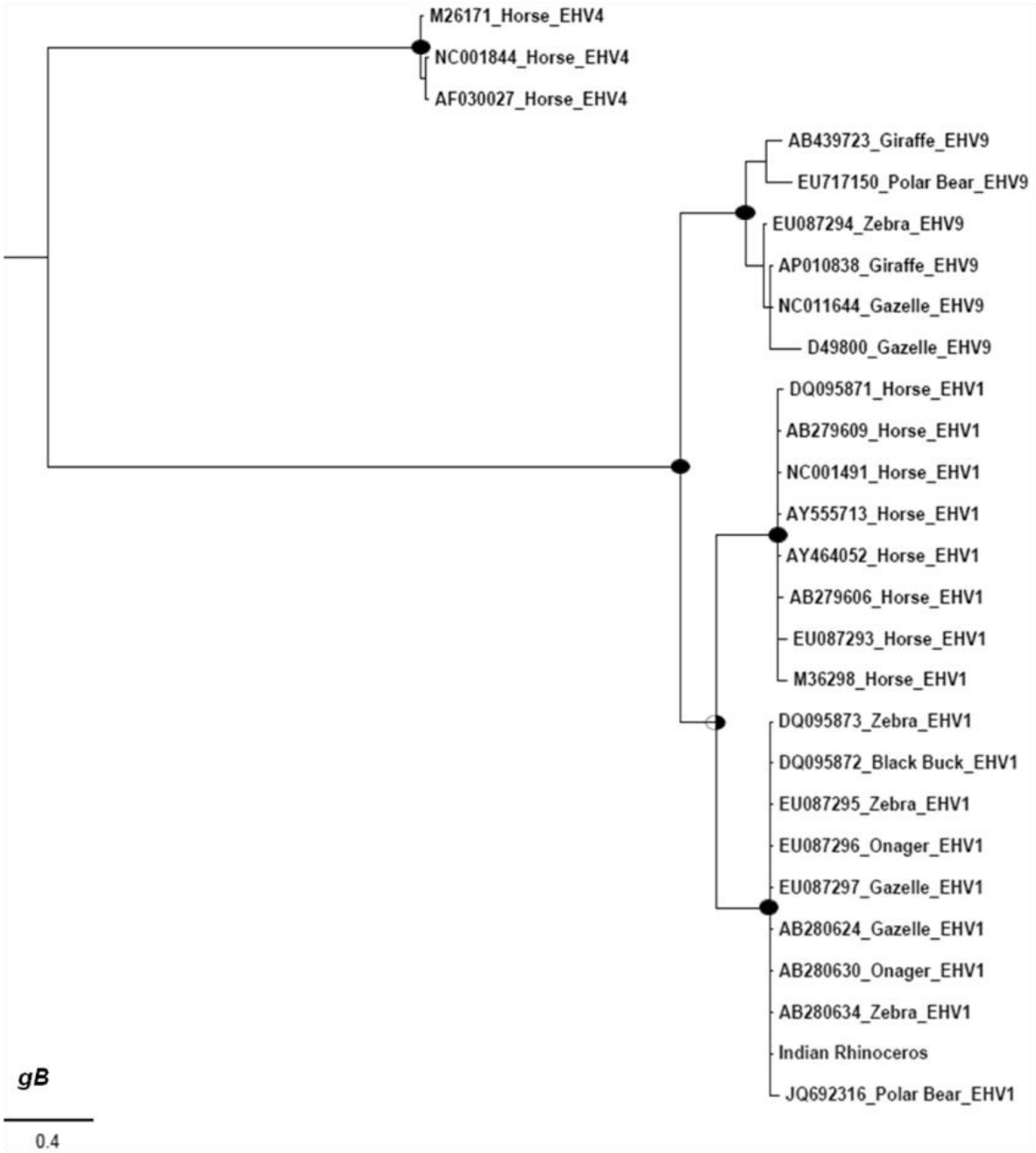
Supplementary Fig. 1



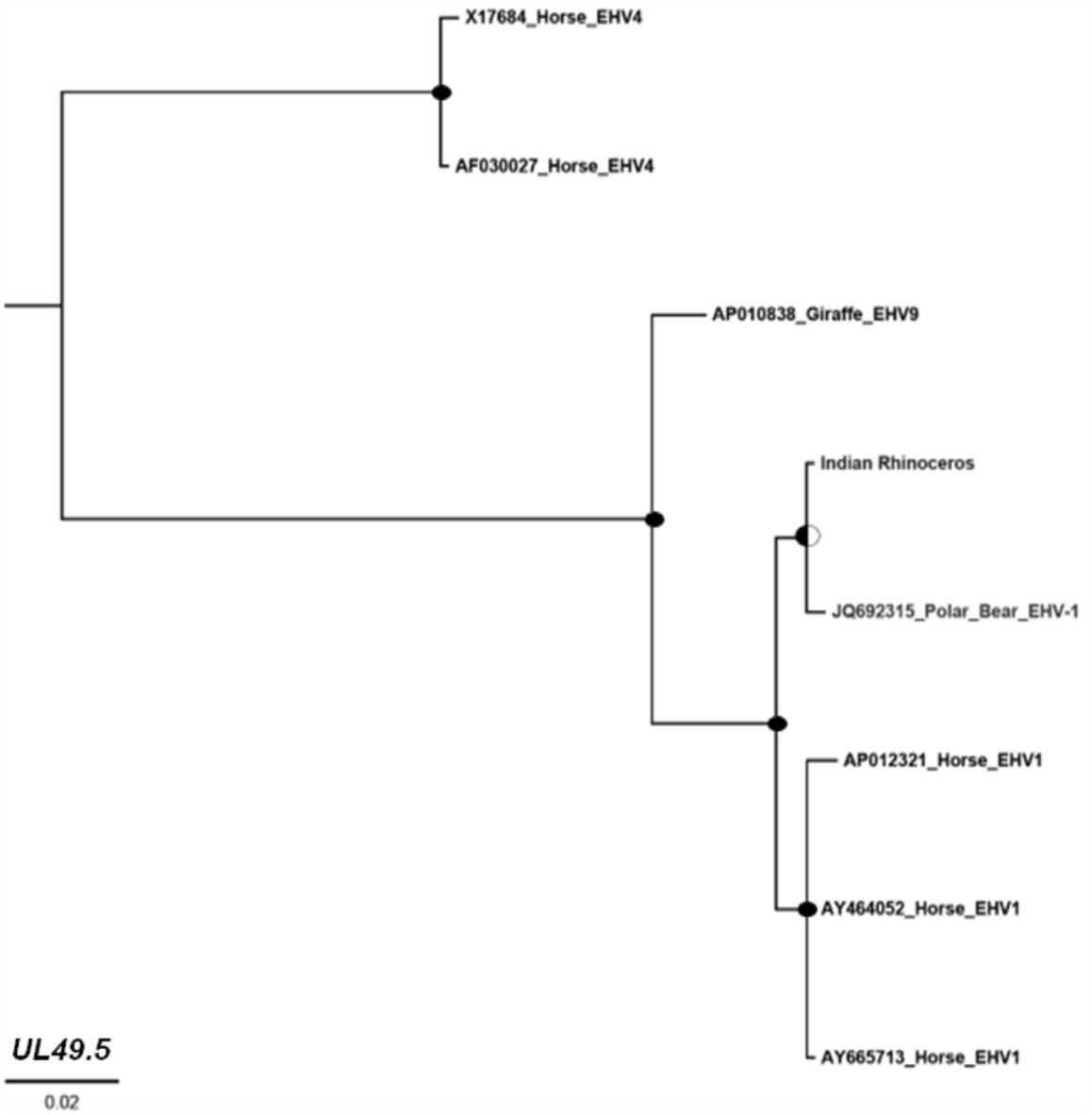
Supplementary Fig. 1. Detection of equine herpesvirus by PCR. PCR products were electrophoresed on a 1 % agarose gel. Wild-type EHV-1 and DNA- and RNA-free water were included as positive (PC) and negative (NC) controls, respectively. A molecular weight marker (Gene Ruler^{TM}Plus DNA Ladder; lane M) was included. Panels A, B, C, D, E and F represent PCR products yielded for a pan-herpes PCR, *gB*, *IR6*, *UL45*, *UL49.5* and *Pol* respectively.

Supplementary Fig. 2A

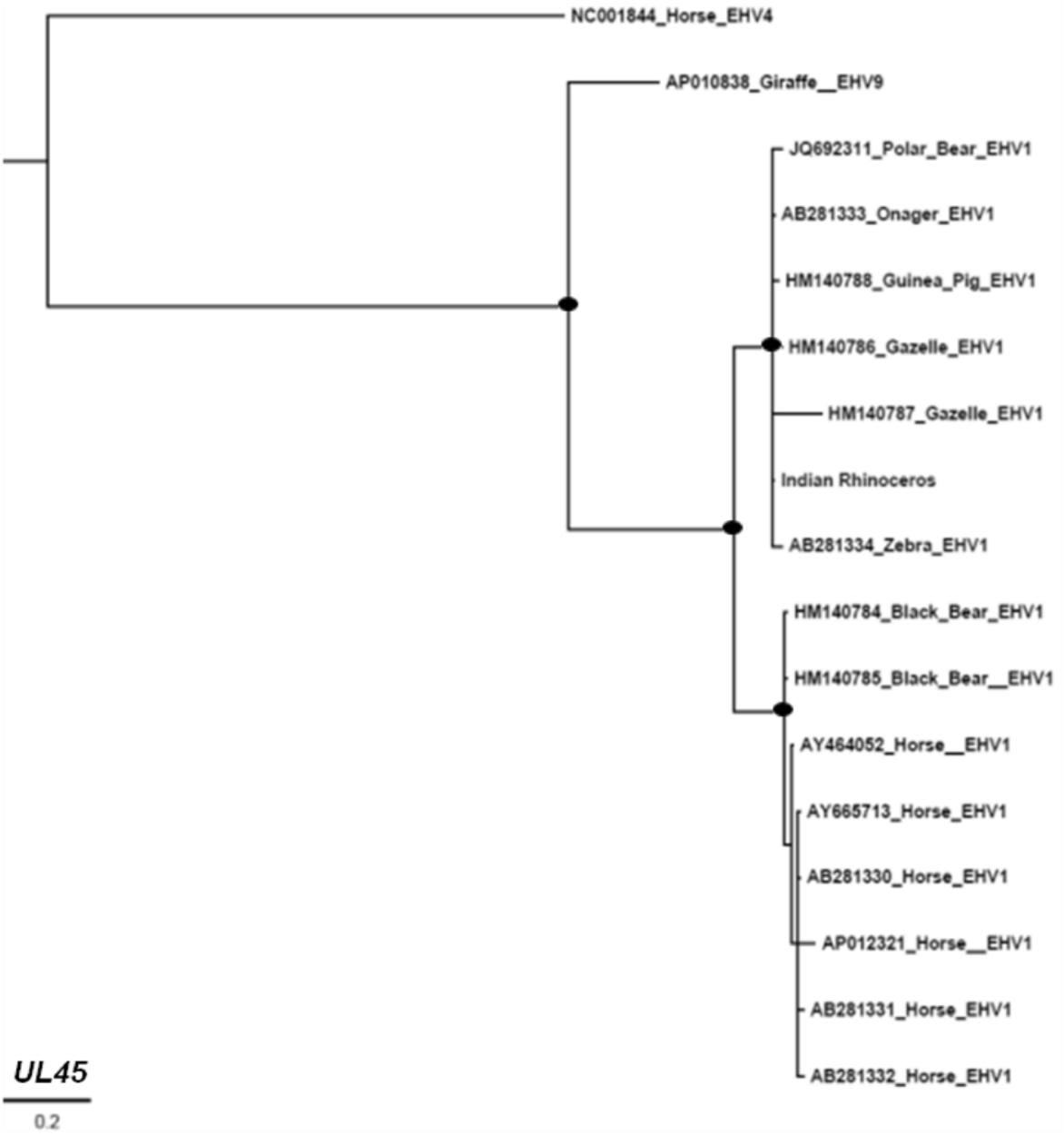
Supplementary Fig. 2B

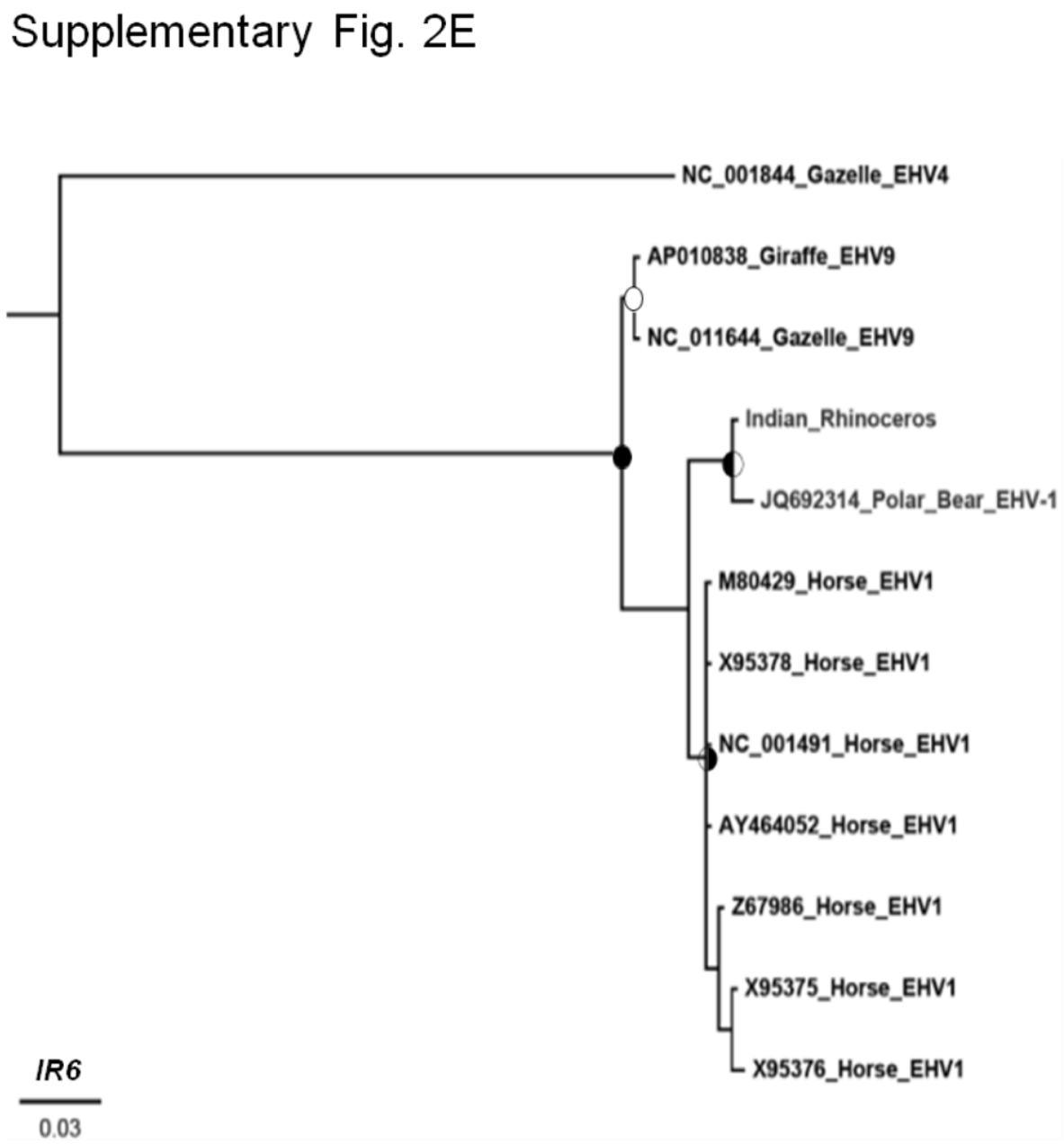


Supplementary Fig. 2C



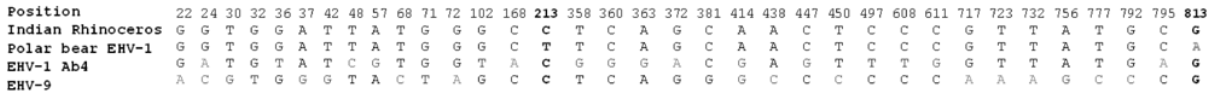
Supplementary Fig. 2D





Supplementary Fig. 2 Phylogenetic relationships of different genes of equine herpesviruses. Maximum Likelihood trees are shown for DNA polymerase (*UL30*) (A), *gB* (B),*UL49.5* (C), *UL45* (D), and *IR6* (E). The Indian rhinoceros is highlighted in red and the other strains, which share the rhinoceros' sequence in the same clade, are highlighted in blue. Node support is indicated by black semicircles for >90% support or white semicircles for <90% but >50% support. The left semicircle represents the posterior probability, and the right semicircle represents the maximum-likelihood bootstrap support. The same trees were obtained regardless of method.

Supplementary Fig. 3



Supplementary Fig. 3 Detection of recombination between EHV-1 and EHV-9 in the DNA polymerase (*UL30)* gene. Analysis of the DNA polymerase gene (*UL30*) isolated from Indian rhinoceros Purana's tissues in a putative EHV-1/EHV-9 recombination region is shown in comparison to a polar bear EHV-1 isolate reported earlier (Greenwood et al., 2012), domestic horse EHV-1 isolate Ab4, and EHV-9. Only variable positions are shown. Positions of each base relative to the first base of the sequence obtained are shown above each column.

Supplementary Table 1. Primer sequences and probes used in this study.

Primer	Sequence	*T*m (c°)	Fragment size (bp)
DFA (F)	5′-GAYTTYGCNAGYYTNTAYCC-3′		
ILK (F)	5′ TCCTGGACAAGCAGCARNYSGCNMTNAA-3′	50	700
KG1 (R)	5′-GTCTTGCTCACCAGNTCNACNCCYTT-3′		
TGV (F)	5′-TGTAACTCGGTGTAYGGNTTYACNGGNGT-3′	50	250
IYG (R)	5′-CACAGAGTCCGTRTCNCCRTADAT-3′		
Pol (F)	5′-TACAACAAAAGATCTACCAG-3′	55	934
Pol(R)	5′-GTGGACGGTACCCCGGAC-3′		
gB (F)	5'-CTTGTGAGATCTAACCGCAC-3′	60	1180
gB (R)	5′-GGGTATAGAGCTTTCATGGGG-3′		
UL49.5(F)	5'-CTACGTCAGTGAGCACACTAT-3′	55	525
UL49.5 (R)	5'-CTTGTAGGTAGAGAGCTAGCC-3′		
IR6(F)	5'-GAAGAGATGAACGAGGGG-3'	60	377
IR6(R)	5'-AATCCTCTTCGGCCCCG-3'		
ORF15 (F)	5'-CGCATCGGTTTCTCTATTACCG-3'	55	709
ORF15 (R)	5'-GTAAAGCAACATGGCAGGAGAC-3'		
qPCR			
gB (F)	5'-CATACGTCCCTGTCCGACAGAT-3'		
gB (R)	5'-GGTACTCGGCCTTTGACGAA-3'		
Probe	5'-6FAMTGAGACCGAAGATCTCCTCCACCGA–BHQ1-3'		

Appendix 1

EHV-1 antigen was detected in Indian rhinoceros lung tissue using Immunohistochemistry.

Immunohistochemistry was performed again using frozen sections obtained from lung tissues. Tissue sections were fixed with a pre-cooled acetone for 10 minutes. The slides were then processed according to the online protocol available on bioLegend website (https://www.biolegend.com/media_assets/support_protocol/BioLegend_IHC_Frozen_121511.pdf).

The anti EHV-1 gB monoclonal antibody (Allen and Yeargan, 1987), was used as the primary antibody at dilutions of 1:200. After application of the secondary antibody (anti-mouse IgG labeled with HRB) (life technologies), the sections were counterstained with 0.05% DAB [3,3'-Diaminobenzidine, 0.015% H_2O_2 in PBS] (Fig. 4.1). EHV-1-negative control (lymph node tissue) obtained from rhinoceros and EHV-1-positive control (lung tissue) obtained from a natural infected horse were used.

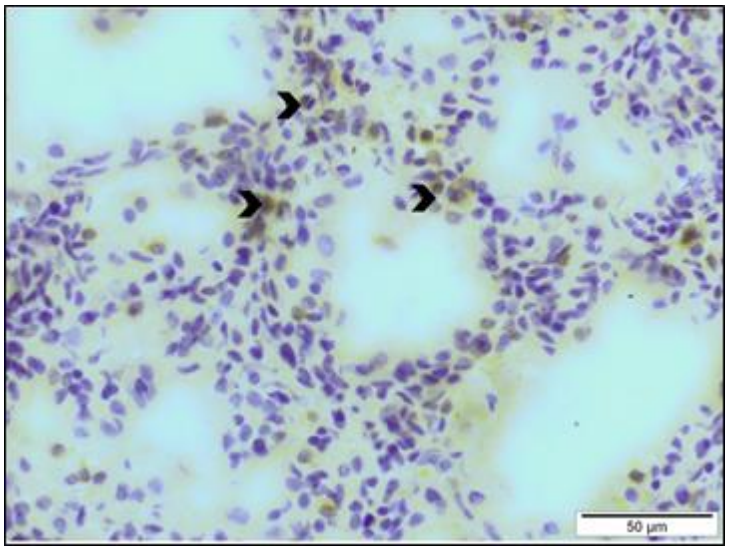


Fig. 4.1 Immunohistochemistry of EHV-1-infected lung. Immunohistologic demonstration of EHV-1 antigen in nuclei of lung epithelium (arrows), using a monoclonal gB primary antibody, and anti-mouse IgG-HRB as a secondary antibody; DAB stain, scale bar = 50 µm.

References

Allen, G.P., Yeargan, M.R., 1987. Use of lambda gt11 and monoclonal antibodies to map the genes for the six major glycoproteins of equine herpesvirus 1. J Virol 61, 2454-2461.

Chapter 5

General discussion and conclusion

Understanding disease emergence requires consideration of the pathogen, host range and ecological interactions that facilitate pathogen survival in nature. The One Health concept considers human, domestic animals and wildlife health to be mutually dependent and should be considered within one ecosystem context (Gilbert *et al.*, 2013).

Among the alphaherpesviruses EHV-1 and EHV-9 have been shown to cross species barriers causing fatalities among non-natural hosts (Chowdhury *et al.*, 1988; Crandell *et al.*, 1988; Fukushi *et al.*, 1997; Kasem *et al.*, 2008; Rebhun *et al.*, 1988; Schrenzel *et al.*, 2008). The rapid lytic cycle and latent persistence with subsequent reactivation constitutes an epidemiological advantage for both viruses. It has been assumed that zebras are the main source of herpesvirus infections in zoos. However, little is known about the current epidemiology of the zebra-derived EHVs.

In this dissertation, I explored the prevalence of EHV-1 and EHV-9 in zoo and wildlife and attempted to answer open questions regarding host range, diversity, and virus recombination. I therefore examined the epidemiology and genetic characterization of EHV-1 and EHV-9 in zoo and wild animals. The work was carried out in three phases. First, I conducted a seroprevalence survey of EHV-1 and EHV-9 in zoo and wild animals to investigate the current epidemiology of both viruses. Second, I characterized the genetic diversity and latency of both viruses in zebras. Third, I investigated the possible existence of a recombinant virus circulating among zoo and wild animals.

Measurement of prevalence is the most frequently way to describe the epidemiology of diseases in natural populations. It is imperative to develop discriminatory methods for diagnostic differentiation between highly similar alphaherpesviruses such as EHV-1 and EHV-9. Therefore, I conducted a seroprevalence survey for EHV-1 and EHV-9 among wide range of captive and wild animals using specific peptide-based ELISA. I was able to discriminate, in particular, the specific antibodies directed against EHV-9 from those against EHV-1. Different families of mammals including *Equidae*, *Rhinocerotidae* and *Bovidae* demonstrated high prevalence of EHV-1 and EHV-9 infections with robust exposure of antibodies for both viruses particularly in wildlife. The higher prevalence of EHVs among zebra populations in the wild compared to zoos suggests captivity may reduce overall viral exposure. However, importation of such silently-infected wild zebras to zoos without proper management and quarantine can result in virus transmission to other zoo animals.

Consequently, caution should be taken by zoos as co-housing zebras with species not naturally sympatric may result in negative outcomes.

The cumulative history of EHV-9 infections suggested zebras as the natural host for EHV-9. However, the high prevalence of EHV-9 antibodies detected in African rhinoceros suggested that they may also act as a reservoir or a secondary host for EHV-9. Thus, even in the absence of zebras, African rhinoceroses may serve as a source of infection. EHV-1 exhibits more variability than EHV-9. The virus diversification, co-infection, and subsequent possible recombination may influence pathogenicity, virulence and host range. All these variables may increase the episode of disease taking into account the growing list of the alphaherpesviruses detected recently in different animal species. Latency is a key aspect of alphaherpesviruses and guarantees viral persistence and makes virus elimination almost impossible. I determined EHV-9 diversity in zoo and wild zebras and investigated the potential of EHV-1 and EHV-9 to establish latency in zebras. Evidence of recombination between different alphaherpesviruses which infect the same species and have high genetic homology has been demonstrated for HSV-1 and HSV-2 and EHV-1 and EHV-4 (Bowden *et al.*, 2004; Javier *et al.*, 1986; Norberg, 2010; Norberg *et al.*, 2004; Pagamjav *et al.*, 2005). The co-occurrence of EHV strains increases the probability of recombination which is mostly considered as a central mechanism of evolution by alphaherpesviruses (Norberg *et al.*, 2015; Thiry *et al.*, 2005). In this thesis, I could detect EHV-1 and EHV-9 co-infection in a zebra which is consistent with the zebra origin of the recombinant viruses detected in polar bear and Indian rhinoceros (Abdelgawad *et al.*, 2014; Greenwood *et al.*, 2012).

Cross species transmission of zebra-borne recombinant EHV-1 among non-equid species was demonstrated in this thesis. DNA sequencing revealed that the Indian rhinoceros which aborted in mid-pregnancy and subsequently was euthanized because of severe neurological disease was infected with the same EHV-1 recombinant strain, which caused severe nervous manifestations and fatality in the polar bear 2010 (Greenwood *et al.*, 2012). This is the first example of a potential chain of infections in captivity from a polar bear to an Indian rhinoceros and extends the host range of zebra-borne EHV-1 to Asian perissodactyls. Indian rhinoceros are highly vulnerable and threatened with extinction (Fischer-Tenhagen *et al.*, 2000). Intensive captive breeding has been undertaken to maintain the species. However, the findings of this thesis raise the question of whether captivity can provide protection against species extinction. In all cases, more precautions and protection measures should be followed by the zoos to avoid mixing of species that are not natural con-specifics with zebras.

The mortality induced by EHV-1 in species that are not con-specifics of zebras may suggest that exposure to the virus may result in particularly severe outcomes for non-African mammals. Thus, being immunological naïve or lack of adaptation to the virus by the new hosts may dictate the course of infection and outcome. Mixing of geographically dispersed mammals regularly occurs in zoological collections and in circuses, which may provide pathogens such as zebra-derived EHV-1 opportunities to disseminate to new hosts and result in severe outcomes.

The main source of EHV-1 transmission between equids is by contaminated fomites. However, in the last episodes of EHVs transmission, the route of transmission is unclear. A live vector, such as rodents, and shared water pools, where the animals gather, could play a role in virus transmission. Further studies should be conducted to investigate these possible sources in disease transmission.

In conclusion, it is becoming clear that zoo populations are at risk of cross-species transmission of EHV-1 and EHV-9 with the taxonomic distribution of affected hosts broadening unabated. The ongoing global threats to the environment increase the importance of zoos to provide animal welfare through good care and a high quality of life. In the context of the control measurements, zoo animals have often come from heterogeneous backgrounds that can influence their ability to cope with certain challenges such as EHV infection. I, therefore, recommend (i) to keep the newly imported-expected natural hosts of EHV-1 and EHV-9 in separate enclosures, away from other species, for at least two weeks before being mixed with other animals. During these two-week quarantines, animals should be inspected for any signs of disease, tested for antibodies against EHV-1 and/or EHV-9, PCR-tested for viral DNA in the blood and nasal swabs. (ii) To prevent introduction of positive-EHVs animals to free-EHVs zoos.

References

Abdelgawad, A., Azab, W., Damiani, A.M., Baumgartner, K., Will, H., Osterrieder, N. and Greenwood, A.D. 2014. Zebra-borne equine herpesvirus type 1 (EHV-1) infection in non-African captive mammals. *Vet Microbiol,* **169**: 102-106.

Bowden, R., Sakaoka, H., Donnelly, P. and Ward, R. 2004. High recombination rate in herpes simplex virus type 1 natural populations suggests significant co-infection. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases,* **4**: 115-123.

Chowdhury, S.I., Ludwig, H. and Buhk, H.J. 1988. Molecular biological characterization of equine herpesvirus type 1 (EHV-1) isolates from ruminant hosts. *Virus Res,* **11**: 127-139.

Crandell, R.A., Ichimura, H. and Kit, S. 1988. Isolation and comparative restriction endonuclease DNA fingerprinting of equine herpesvirus-1 from cattle. *American journal of veterinary research,* **49**: 1807-1813.

Fischer-Tenhagen, C., Hamblin, C., Quandt, S. and Frolich, K. 2000. Serosurvey for selected infectious disease agents in free-ranging black and white rhinoceros in Africa. *Journal of wildlife diseases,* **36**: 316-323.

Fukushi, H., Tomita, T., Taniguchi, A., Ochiai, Y., Kirisawa, R., Matsumura, T., Yanai, T., Masegi, T., Yamaguchi, T. and Hirai, K. 1997. Gazelle herpesvirus 1: a new neurotropic herpesvirus immunologically related to equine herpesvirus 1. *Virology,* **227**: 34-44.

Gilbert, A.T., Fooks, A.R., Hayman, D.T., Horton, D.L., Muller, T., Plowright, R., Peel, A.J., Bowen, R., Wood, J.L., Mills, J., Cunningham, A.A. and Rupprecht, C.E. 2013. Deciphering serology to understand the ecology of infectious diseases in wildlife. *EcoHealth,* **10**: 298-313.

Greenwood, A.D., Tsangaras, K., Ho, S.Y., Szentiks, C.A., Nikolin, V.M., Ma, G., Damiani, A., East, M.L., Lawrenz, A., Hofer, H. and Osterrieder, N. 2012. A potentially fatal mix of herpes in zoos. *Curr Biol,* **22**: 1727-1731.

Javier, R.T., Sedarati, F. and Stevens, J.G. 1986. Two avirulent herpes simplex viruses generate lethal recombinants in vivo. *Science,* **234**: 746-748.

Kasem, S., Yamada, S., Kiupel, M., Woodruff, M., Ohya, K. and Fukushi, H. 2008. Equine herpesvirus type 9 in giraffe with encephalitis. *Emerg Infect Dis,* **14**: 1948-1949.

Norberg, P. 2010. Divergence and genotyping of human alpha-herpesviruses: an overview. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases,* **10**: 14-25.

Norberg, P., Bergstrom, T., Rekabdar, E., Lindh, M. and Liljeqvist, J.A. 2004. Phylogenetic analysis of clinical herpes simplex virus type 1 isolates identified three genetic groups and recombinant viruses. *J Virol,* **78**: 10755-10764.

Norberg, P., Depledge, D.P., Kundu, S., Atkinson, C., Brown, J., Haque, T., Hussaini, Y., MacMahon, E., Molyneaux, P., Papaevangelou, V., Sengupta, N., Koay, E.S., Tang, J.W., Underhill, G.S., Grahn, A., Studahl, M., Breuer, J. and Bergstrom, T. 2015. Recombination of Globally Circulating Varicella-Zoster Virus. *J Virol,* **89**: 7133-7146.

Pagamjav, O., Sakata, T., Matsumura, T., Yamaguchi, T. and Fukushi, H. 2005. Natural recombinant between equine herpesviruses 1 and 4 in the ICP4 gene. *Microbiology and immunology,* **49**: 167-179.

Rebhun, W.C., Jenkins, D.H., Riis, R.C., Dill, S.G., Dubovi, E.J. and Torres, A. 1988. An epizootic of blindness and encephalitis associated with a herpesvirus indistinguishable from equine herpesvirus I in a herd of alpacas and llamas. *J Am Vet Med Assoc,* **192**: 953-956.

Schrenzel, M.D., Tucker, T.A., Donovan, T.A., Busch, M.D., Wise, A.G., Maes, R.K. and Kiupel, M. 2008. New hosts for equine herpesvirus 9. *Emerg Infect Dis,* **14**: 1616-1619.

Thiry, E., Meurens, F., Muylkens, B., McVoy, M., Gogev, S., Thiry, J., Vanderplasschen, A., Epstein, A., Keil, G. and Schynts, F. 2005. Recombination in alphaherpesviruses. *Reviews in medical virology,* **15**: 89-103.

Summary

Although EHV-1 infections are mainly restricted to equine species, there is increasing evidence that EHV-1 can cross species barriers and spread beyond its natural equid hosts. EHV-9 is the recently discovered virus strain of equine herpesviruses with an unknown natural host. Both EHV-1 and EHV-9 infections of non-definitive hosts often result in neuronal infection with high case fatality rates. However, the questions remain: what the natural host(s) and the complete host range of both viruses are? It becomes urgent to answer these questions after a series of fatal infections which have been detected in the last few years. Detection of a new recombinant EHV-1/EHV-9 virus strain with fatalities in polar bear and Indian rhinoceros requires further investigation regarding the origin of the recombinant virus. Cumulative data suggested that the recombinant viruses originated in zebras. However, it is still unclear whether recombination occurred in captive zebras or in wild zebras. Similarly, it is unclear whether recombination is a rare or frequent event in zebras. The seroprevalence survey conducted in this thesis demonstrated that EHV-1 and EHV-9 have evolved a broad host range among African mammals including distantly related perissodactyls. Although the high prevalence of EHV-9 in African rhinoceros suggested that this species could be a reservoir or secondary host to the virus, molecular studies showed the ability of both EHV-1 and EHV-9 to induce infection in zebra with the ability of EHV-9 to establish latency. The co-occurrence of EHV-1 and EHV-9 in the same zebra supports the hypothesis that zebras are a natural host for both viruses where recombination can take place. However, one cannot exclude the role of African rhinoceros in virus transmission. Further molecular studies to investigate different viral strains circulating in African rhinoceros populations and whether they differ from zebra isolates are required.

Deaths among animals infected with recombinant EHV-1 strains were reported in Indian rhinoceros and polar bear. It is worth noting that wild non-African animal species never come in contact with wild African animals, which may explain the relatively frequent observed fatal disease in species that are not naturally sympatric with African Perissodactyls.

According to International Union for Conservation of Nature and Natural Resources (IUCN), the five extant rhinoceros species (black, white, Indian, Javan and Sumatran) are listed as critically endangered species. Since diseases can contribute to population declines and ultimately lead to species extinctions, attention should be paid to such potentially emerging EHV infections. It is, therefore, critical to pay attention to EHV-1 and its relatives as newly emerging infectious agents, particularly in zoos that can provide an environment for mixing different species and as such provide opportunities for viruses to acquire new species.

Zusammenfassung

Equine Herpesviren Typ 1 und 9 (EHV-1 und -9) Prävalenz und Biologie in Zoo und Wildtieren

Obwohl EHV-1-Infektionen hauptsächlich auf equine Arten begrenzt sind, gibt es immer mehr Hinweise darauf, dass EHV-1 Artgrenzen überwinden und sich somit auch außerhalb seines equinen Wirtes ausbreiten kann. EHV-9 wurde erst vor kurzem als Virusstamm der equinen Herpesviren entdeckt und der natürliche Wirt ist noch nicht bekannt. Sowohl EHV-1- als auch EHV-9-Infektionen verursachen in nicht-vorgesehenen Wirtstieren häufig neuronale Infektionen mit einer hohen Mortalitätsrate. Es bleibt die Frage, welche natürlichen Wirte und welches Spektrum an Wirten die beiden Virusstämme haben? Nachdem eine Reihe tödlicher Infektionen in den letzten Jahren aufgetreten ist, wird es immer wichtiger diese Frage zu beantworten. Die Entdeckung eines neuen rekombinanten EHV-1/EHV-9-Virustammes, welches zum Tod eines Eisbären und eines Indischen Panzernashornes führte, erfordert weitere Studien bezüglich der Herkunft dieses rekombinanten Virus. Die gesammelten Daten weisen darauf hin, dass die rekombinanten Viren von Zebras stammen. Allerdings ist noch unklar, ob die Rekombination in gefangenen oder wilden Zebras stattgefunden hat und ob es sich bei der Rekombination um ein seltenes oder eher häufiges Ereignis in Zebras handelt. Die in dieser Arbeit durchgeführte Seroprävalenz-Studie zeigte, dass sich EHV-1 und EHV-9 in einem breiten Wirtsspektrum von afrikanischen Säugetieren einschließlich der entfernt verwandten Unpaarhufer (Perissodactyla) entwickelt haben. Obwohl die hohe Prävalenz von EHV-9 in afrikanischen Nashörnern vermuten lässt, dass es sich bei dieser Art um ein Reservoir oder sekundären Wirt für dieses Virus handeln könnte, haben molekulare Studien gezeigt, dass sowohl EHV-1 als auch EHV-9 in der Lage sind Infektionen in Zebras zu verursachen. Das Vorkommen der beiden Virusstämme EHV-1 und EHV-9 im selben Zebra unterstützt die Hypothese, dass Zebras der natürliche Wirt beider Virusstämme sind und hier eine Rekombination stattfinden kann. Jedoch kann die Rolle von afrikanischen Nashörnern in der Virusübertragung nicht ausgeschlossen werden. Es sind weitere molekulare Studien nötig, in denen verschiedene Virusstämme in afrikanischen Nashornpopulationen untersucht werden und zu denen in Zebras verglichen werden.

Es gibt Berichte von Todesfällen in einem Indischen Panzernashorn und einem Eisbären, die mit einem rekombinanten EHV-1-Stamm infiziert waren. Hierbei ist wichtig zu erwähnen, dass nicht-afrikanische Tierarten in der Natur niemals in Kontakt mit wilden,

afrikanischen Tieren kommen. Dies könnte den relativ häufig tödlichen Verlauf in Tierarten, die nicht sympatrisch mit afrikanischen Unpaarhufern sind, erklären.

Laut der „International Union for Conservation of Nature and Natural Resources (IUCN)" sind die fünf bestehenden Nashornarten (Spitzmaulnashorn, Breitmaulnashorn, Indisches Panzernashorn, Java-Nashorn und Sumatra-Nashorn) vom Aussterben bedroht. Seit Krankheiten zu der Reduktion einer Population beitragen können und im schlimmsten Fall eine Art sogar auslöschen können, sollte man vermehrt solchen neu auftretenden Virus-Infektionen Aufmerksamkeit schenken. Daher ist es besonders wichtig, EHV-1 und seine Derivate als neue Krankheitserreger anzusehen, vor allem in Zoos, die einen Lebensraum für viele verschiedene Tierarten darstellen und es somit Viren ermöglichen, neue Wirtstiere zu erhalten.

List of publications

1. Abdelgawad A, Hermes R, Damiani A, Lamglait B, Czirják GÁ, East M, Aschenborn O, Wenker C, Kasem S, Osterrieder N, Greenwood AD. Comprehensive Serology Based on a Peptide ELISA to Assess the Prevalence of Closely Related Equine Herpesviruses in Zoo and Wild Animals. PLoS One. 2015 Sep 17; 10(9):e0138370. doi: 10.1371/journal.pone.0138370. eCollection 2015.

2. Azza Abdelgawad, Walid Azab, Armando M. Damiani, Katrin Baumgartner, Hermann Will, Nikolaus Osterrieder, Alex D. Greenwood, Zebra-borne equine herpesvirus type 1 (EHV-1) infection in non- African captive mammals. Vet. Microbiol. (2014).

3. Azab W, El-Sheikh A, **Abdel-Gawad Azza**. In vitro characterization of EHV-4 gG-deleted mutant. Virus Genes. 2012 Feb;44(1):109-11.

4. Walid Azab, Kentaro Kato, **Azza Abdel-Gawad**, Yukinobu Tohya, and Hiroomi Akashi. Equine Herpesvirus 4: Recent Advances Using BAC Technology. Vet Microbiol. 2011 May 12; 150(1-2):1-14.

Acknowledgements

Turning aspiration into reality is very hard, unless you are lucky enough to be surrounded by people who always help you to achieve your goals.

First, I would like to express my sincere gratitude and great appreciation to my supervisor Prof. Alex Greenwood for supervising, supporting, and guiding me during my PhD. You were a great and patient teacher, and it was a great pleasure to work with you.

I am thankful to my mentors, Prof. Nikolaus Osterrieder and Dr. Marion East, who provided me with time for discussion and supportive feedback through the DRS meetings.

I would like to thank Prof. Heribert Hofer for giving me the opportunity to conduct my project at the IZW.

My special thanks to all the co-authors in the manuscripts of this thesis, especially to all members of zoos who provided me with samples; especially, Dr. Benjamin Lamglait, Réserve Africaine de Sigean (France), Dr. Alexis Lecu, PARC Zoologique de Paris (France), Dr. Arne Lawrenz, Wuppertal Zoo (Germany), Dr. Christine Gohl, Münchner Tierpark Hellabrunn (Germany), Dr. Claudia Szentiks, IZW (Germany), Dr. Dorothée Ordonneau, CERZA Lisieux Zoo (France), Dr. Gabby Drake, Chester Zoo (UK), Dr. Gudrun Wibbelt, IZW (Germany), Dr. Günter Strauss, Tierpark Berlin (Germany), Dr. Julia Gräger, Zoo Hannover (Germany), Dr. Katrin Baumgartner, Nuremberg Zoo (Germany), Dr. Christian Wenker, Zoo Basel (Switzerland), Dr. Tobias Witzens, Wilhelma der Zoologisch-Botanische Garten (Germany) and Dr. Ulrike Rademacher, EAZA Equid TAG.

I wish to express my thanks to all the members of the wildlife diseases department at the IZW; especially to my fellow PhD students Niccolo Alfano and Marina Escalera for all the nice moments.

I want to thank Karin Hoenig, IZW and Sebastian Bischofberger, Institute of Virology, FU for technical support.

I want to thank Else Neumann scholarship, FU, Berlin (NaFöG) for their support. Furthermore, I would like to thank the coordinator Angela Daberkow of the Dahlem Graduate School for her effort and help to obtain my PhD.

I would like to express my gratitude and thanks to my parents and sisters.

Finally, I want to thank and dedicate this thesis to the most important people in my life; my husband and my three children for their love, patience, support, and continuous encouragement to complete this work.

Collaborations and contributions

1- Institut für Virologie, Robert von Ostertag-Haus, Zentrum für Infektionsmedizin, Freie Universität Berlin, Berlin, Germany

2- European zoos provided us with samples required for the study:
Réserve Africaine de Sigean (France), PARC Zoologique de Paris (France), Wuppertal Zoo (Germany), Münchner Tierpark Hellabrunn (Germany), CERZA Lisieux Zoo, (France), Chester Zoo (UK), Tier park Berlin (Germany), Zoo Hannover (Germany _ Nuremberg Zoo (Germany), Zoo Basel (Switzerland), Wilhelma der Zoologisch Botanische Garten (Germany), EAZA Equid TAG.

3- Prof. Simon Y.W. Ho and Dr. Kyriakos Tsangaras helped in phylogenetic analyses.

Selbständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Doktorarbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe.

I hereby confirm that I did all this work myself.

Azza Abdelgawad
September 12th, 2016