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Seasonal host and ecological drivers may promote restricted water as a viral vector

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Abstract

In climates with seasonally limited precipitation, terrestrial animals congregate at high densities at scarce water sources. We hypothesize that viruses can exploit the recurrence of these diverse animal congregations to spread. In this paper, we test the central prediction of this hypothesis -- that viruses employing this transmission strategy remain stable and infectious in water. Equid herpesviruses (EHVs) were chosen as a model as they have been shown to remain stable and infectious in water for weeks under laboratory conditions. Using fecal data from wild equids from a previous study, we establish that EHVs are shed more frequently by their hosts during the dry season, increasing the probability of water source contamination with EHV. We document the presence of several strains of EHVs present in high genome copy number from the surface water and sediments of waterholes sampled

across a variety of mammalian assemblages, locations, temperatures and pH. Phylogenetic analysis reveals that the different EHV strains found exhibit little divergence despite representing ancient lineages. We employed molecular approaches to show that EHV₁ shed remain stable in waterholes with detection decreasing with increasing temperature in sediments. Infectivity experiments using cell culture reveals that EHV₁ remain infectious in water derived from waterholes. The results are supportive of water as an abiotic viral vector for EHV₁.

Introduction

Freshwater distribution varies geographically and seasonally, with some regions experiencing extreme seasonal shortages such as in East Africa and Central Asia during the dry season. The majority of mammals have a minimum daily water requirement to survive and in seasonally water-limited environments, surface water dependency results in seasonal mass migrations where frequent and unstable congregations of animals are observed^{1,2}. During times of water restriction, there is intensified forage depletion and increased competition for water among all species, increasing contact rates of animals². Seasonal fluctuation of surface water availability may therefore influence waterborne pathogen transmission.

Seasonal surface water represents a recurring opportunity for pathogens to infect multiple hosts^{3,4}. Viruses, however, are obligate intracellular parasites and cannot replicate under abiotic conditions. Surface water is generally considered a hostile environment for mammalian viruses with lipid envelopes⁵. Despite these biochemical constraints, many viruses are capable of remaining stable and infectious in water, such as human rota-, astro-, noro- and influenza viruses⁶⁻⁹ and animal viral pathogens^{10,11}. These viruses enter the environmental water either through livestock and wildlife excretion or in urban areas as a by-product of water management such as shared plumbing^{12,13}. Nevertheless, water is not the main vector for these viruses and transmission generally involves direct contact with infectious exudate or fecal-oral transmission¹². In such cases, transmission in water is indirect due to features viruses have evolved to support transmission by other routes making them water stable¹⁴.

The objective of the current study was to determine if viral stability is sufficient for some mammalian viruses to use direct infection from water in order to increase host infection rates in natural environments during seasonal water shortages. As a model, we examined equid herpesvirus (EHV₁) stability and infectivity in environmental water. EHV₁ were selected as

they are known to remain stable and infectious in water for weeks under laboratory conditions and circulate in wildlife in both Africa and Mongolia ¹⁵.

We have sampled surface water and sediments from Tanzania, Namibia and Mongolia and subsequently screened them for EHV. All regions sampled experience regular seasonal water shortages and migratory animals that often result in massive mixed species congregations (Figure 1). They are home to various species of migratory equids – the primary host for EHV. In the Etosha National Park in Namibia, two zebra species are present, the mountain zebra (*Equus zebra*) and the plains zebra (*Equus quagga*), while the Serengeti National Park in Tanzania has only the latter. The great Gobi Desert in Mongolia has two wild equid species, the wild Przewalski's horse (*Equus ferus przewalskii*), the Asiatic wild ass (*Equus hemionus hemionus*), and large numbers of domestic horses (*Equus caballus*) ¹⁶.

Our study tests three main hypotheses: 1) EHV remains infectious in water and sediment over a prolonged period of time under natural conditions; 2) EHV hosts increase viral shedding during times of seasonal water shortages; and 3) using water as an abiotic vector may restrain EHV strain divergence.

Methods

Collection of sediment, faecal and water samples

Samples were collected in Namibia from Etosha National Park and farmland in November 2015. Samples from Tanzania were collected in February, June, July and October 2016 from the Serengeti National Park. Mongolian samples were collected from South East Gobi in October 2015 and Great Gobi B Strictly Protected Area (SPA) in June and July 2016 (**Supplementary Table 1 and 2**). Twenty-five grams of the first 1-3 cm of wet sediment were collected. Fifty ml of water was passed through 0.22 µm Sterivex filters (Millipore, USA) using disposable 50-ml syringes. Subsequently, samples were stored on ice and frozen at -20 °C at the field stations (within 6 h of collection). Temperature and pH were recorded at each site. Faecal samples were collected opportunistically from 270 individuals in two distinct habitat types: 1) the short grass plains in the south of the Serengeti National Park where zebras typically occur during the rainy season, and 2) the open woodland savannah with long grass in the center of the park, where zebras were sampled during the dry season. For each sample the sex, age class and in mature individuals, reproductive state were recorded ¹⁷. Subsamples for DNA extraction were stored in RNAlater (Sigma-Aldrich, Taufkirchen, Germany) in the field and were frozen at -20 °C for shipment and storage.

Preparation of water filtrate samples

Thirty-two ml of water filtrate was ultra-centrifuged at 28,000 rpm for 2 hours to pellet DNA and viral particles in each sample. The filtrate was removed. the pellet re-suspended in 1 ml of cold phosphate-buffered saline (PBS) (pH 7.2) (Sigma-Aldrich, USA), and stored overnight at 4 °C then subsequently stored at -80 °C.

RNA/DNA isolation of centrifuged water filtrate and sediment

Five-hundred microliters of centrifuged water filtrate was DNA extracted using the RTP[®] DNA/RNA Virus Mini Kit (Stratec biomedical. Germany) with the following modifications: 400 µl of lysis buffer, 400 µl of binding buffer and 20 µl of proteinase K and carrier RNA were used per sample. Samples were eluted in 60 µl of elution buffer.

DNA was extracted from soil using the NucleoSpin Soil kit (Macherey-Nagel, Germany).

Five- hundred milligrams of soil was extracted using an elution volume of 100 µl.

cDNA was generated from RNA extracts using the Invitrogen SuperScript IV (Thermo Fisher Scientific, USA). Second-strand synthesis was carried out by adding 1 µl of Klenow DNA polymerase I (Thermo Fisher Scientific, USA) to 20 µl of cDNA and incubated at 37 °C for 60 min and then at 75 °C for 20 min. cDNA concentrations were determined with an Agilent TapeStation (Agilent Technologies USA) using D1000 ScreenTapes and reagents.

Screening of samples for EHV

EHV screening from water, fecal and sediment employed a nested pan-herpes PCR¹⁸. The resulting amplicons were Sanger-sequenced. Positive EHV samples were used for cell culture.

Screening of fecal samples for EHV and fecal glucocorticoid metabolites (fGCM)

The protocol and data for fCCM and fecal PCRs were originally published in Seeber *et al*, 2018¹⁷. In brief, EHV faeces screening of free-ranging plains zebras in the Serengeti National Park was carried out as previously described¹⁹. DNA was extracted using a NucleoSpin soil kit (Macherey-Nagel). Subsamples for fGCM extraction were stored frozen, and laboratory procedures and fGCM measurement using an enzyme immunoassay targeting 11β hydroxyetiocholanolone were conducted as described previously¹⁷.

Cell culture

Rabbit kidney -13 cell (RK-13), bovine kidney (MDBK). and equine dermal cell (ED) monolayers were grown at concentrations of 1×10^7 cells per ml (Table 1) as described Dayaram *et al*¹⁵. Four hundred µl (2×10^5 cells/ml) was added to each well of 24-well plates with positive (EHV-1 strain Ab4) and negative controls (sterile H₂O) included. Plates were incubated at 37 °C for 2 hours to allow for cell adherence. Subsequently, 150 µl of sample filtrate was added to the well. Two wells per sample for each cell line were inoculated.

Samples cultures were further incubated at 37 °C under 5% CO₂ for 5 days. The samples were passaged and incubated for 5 more days at 37 °C under 5% CO₂. Plates were then stained with 10% formalin and 1% crystal violet and examined by microscopy for cytopathic effects (CPE). Cells were scraped from plates and five hundred µl of cell culture samples were frozen at -80 °C. DNA was extracted from the remaining 500 µl of cell culture.

DNA extraction

Infected cells were ruptured by freeze thaw cycles. Cells and cellular debris were pelleted by centrifugation (5,000 rpm for 2 min). Viral DNA was extracted from 500 µl of cell culture using the RTP[®] DNA/RNA Virus Mini Kit (Strattec biomedical, Germany) and eluted in 65 µl of elution buffer.

Quantitative PCR (qPCR) for detection of EHV-1, EHV-2 and EHV-5

EHV-1 presence and genome copy number (GCN) was determined from water filtrate when CPE was observed. The qPCRs targeted glycoprotein B (gB) of EHV-1 (106 bp) (GenBank accession no. M36298), EHV-2 (174 bp) (GenBank accession no. NP_042604) and EHV-5 (297 bp) (GenBank accession no. NC_026421.1)²⁰⁻²². Sample values were regressed to the slope of a standard curve generated from serial dilutions of the: isolated DNA from EHV-1 BAC, Ab4 strain²³, 174 bp EHV-2 gBlock[®] Gene fragment generated from the gB gene of EHV-2, and a 297 bp EHV-5 gBlock[®] Gene fragment generated from the EHV-5 gB gene.

PCR confirmation of Jaagsiekte sheep retrovirus (JSRV)

Nested PCR for JSRV confirmed its presence in Tanzanian cell culture samples and absence in negative control culture wells²⁴. The ~176 bp PCR product was Sanger sequenced (LCG genomics).

PacBio sequencing

Viral DNA extracted from RK-13 and MDBK cell culture samples positive for CPE was quantified using the Agilent TapeStation (**Supplementary Table 2**). DNA was diluted in 100 µl sterile water and fragmented in Covaris miniTUBE Blue 3.0 kb (Covaris, USA) to an average size of 3-4 kb following the Covaris M220 focused-ultasonicator protocol.

Cell culture fragmented DNA samples were individually purified using AMPure XP beads (Beckman Coulter, USA), and prepared as sequencing libraries using the PacBio (Pacific Biosciences, USA) 5 kb Template Prep Protocol and the SMRTbell[™] Template Prep Kit 1.0 (Max Delbrück Centre, Germany). The PacBio RS II platform was used²⁵.

Assembly of the EHV genomes

PacBio RS II sequences were aligned to Equine Herpesvirus 1 strain V592 (AY464052) using Geneious Prime 2019.1.3 read mapper and confirmed with BLASR long read aligner

(PacBio). Mapping quality was manually determined using Geneious prime. Consensus EHV-1 sequence for samples W6_16683 and W20_16681 were generated from the mapped reads (SRA accession: PRJNA577578).

Illumina libraries

Extracted DNA and cDNA from JSRV positive cultures, water and sediment and were fragmented to an average of 350 bp using the Covaris M220 (Covaris, USA). Illumina libraries were built as previously described^{26,27} with adaptations^{28,29}.

Viral and EHV full genome hybridization capture

Fourteen samples from Tanzania (7 from water and 7 from sediment) and seven samples from Mongolia (4 sediment and 3 water) were tested. Baits for capturing known viruses were designed based on the pan-viral microarray Virochip³⁰. Baits for EHV full genome capture were tiled based on all EHV genomes available in GenBank as of the 24th of October 2017. The baits were synthesized by Arbor Biosciences using MYbaits® Custom target enrichment for high throughput sequencing with the following protocol adjustments: samples were pooled in groups of two, cDNA and DNA were pooled separately. 5 µl of baits per pooled sample was used. The cleaned captured libraries were amplified using Herculase II fusion polymerase (Agilent Technologies, USA) as previously described²⁹. The indexed amplified libraries were pooled for paired-end sequencing on the Illumina MiSeq platform.

Sequence analysis

Raw Illumina sequences were processed using Cutadapt for quality trimming and adaptor removal³¹. Curated fastq files were further processed using Viral Identification Pipeline (VIP) with the VIP sense option to screen for viral sequences³². VIP identified five EHV positive samples. All samples including VIP positive ones were mapped to the EHV-1 strain V592 (AY464052) reference genome using Burrows-Wheeler Aligner (BWA-MEM version 0.7.17r1188)³³. Alignments were sorted and de-duplicated using samtools version 1.9³⁴. Geneious Prime was used to generate consensus sequences from the alignments (SRA accession: PRJNA577578).

Phylogenetic analysis was performed PacBio data for sample W06 and W20 and five hybridization capture sequences. Phylogenetic analysis for glycoprotein B and DNA polymerase genes for sample W20 were performed. Alignments were performed using MUSCLE³⁵ and MAFFT³⁶. The near-full genome tree used a trimmed dataset where sequences near 99-100% identity were represented by a single sequence. Because of the lack of available full EHV-1 genomes, sequences were trimmed to 100 KB to include more strains in the analysis. Genes that were 100% identical (NCBI data base as of November

2018) were removed with one sequence selected to represent a given strain. Alignments were used to produce approximate maximum-likelihood phylogenetic guide trees as described in ³⁷.

Statistical analyses of pH and temperature on EHV detection

To assess whether temperature and pH correlated with the detection of EHV in water sources we ran logistic regression models, separately for EHV detection in water and sediment samples in R, using spaMM version 3.1 ³⁸. As predictor variables, we included pH and temperature as continuous fixed effects and we accounted for the effect of the spatial dependence between observations using the traditional Matérn correlation structure. Significance of fixed effects was tested by comparing the likelihood ratio statistic to its distribution under the null hypothesis. We generated the latter by drawing 2,000 parametric bootstrap replicates.

Statistical analyses of EHV shedding

Data on equid health including individual stress hormone levels, aggregation size, sex, age and reproductive status was collected in tandem with water samples, and results were published previously ^{17,19} (**Supplementary table 4**). The data was then combined with the current study to perform statistical analysis to determine if EHV presence in faecal samples correlated with water availability, individual stress hormone level, aggregation size, sex, age and reproductive status. To assess whether seasonal variation in drinking water correlated with the detection of EHV in zebra faecal samples a logistic regression model was run in R. Sampling location (short grass plain vs. open woodland savannah) was the main predictor value, which served as a proxy for drinking water availability. Additional predictors included aggregation size (small, medium, large), faecal glucocorticoid metabolites (fGCM) and age-sex-reproduction class (immature females, immature males, adult lactating females, adult pregnant females, adult other females, adult bachelor males, adult harem males). Significance of predictors was tested by using likelihood ratio tests comparing the full model to a reduced one in which the respective predictor was removed.

Results

Detection of EHV in samples from seasonal waterholes

EHV presence was confirmed by PCR in sediments and surface water sampled in Tanzania, Namibia, and Mongolia (**Figure 1**), demonstrating that animals utilizing the waterholes shed virus. Viral genome copy number (GCN) for EHV-1, -2 and -5 of water filtrate and sediment samples was determined by qPCR demonstrating contamination of the water with EHV (**Table 1**).

EHVs were found more often in sediment than in the water as determined by pan-herpes PCR (sediment n=20, water filtrate n=16). The pan-herpes PCR results were generally supported by the qPCR results (**Supplementary Table 1 and 2**); however, qPCRs could better differentiate among virus species and samples where multiple species were present (**Table 1**).

Mongolia had the most positive samples as determined by qPCR (n=35) and pan-herpes PCR (n=21). qPCR also showed that EHV-1, -2 and -5 were present in both sample types (water filtrate n= 23, sediment n= 32), however, higher GCN for all EHV types were observed in the sediment samples with some sample from Mongolia having up to 290 GCN of EHV-2 (**Table 1**).

EHV-1 and Jaagsiekte sheep retrovirus (JSRV) remain stable and infectious in environmental water

Cell culture confirmed that EHV-1 remained infectious in environmental water. We selected water samples for culture that were positive for EHV-1 in the pan-herpes PCR. Culture from sediment was attempted but failed due to other microorganisms present in the samples.

Therefore, only water filtrate was used for subsequent cell culture isolation attempts.

Of the 8 filtrate samples from Mongolia cultured on three different cell lines, four produced cytopathic effects after 10 days in both RK-13 and bovine kidney (MDBK) cell lines (**Table 2**). Three samples which had low GCN of EHV-1 DNA determined by qPCR failed to culture. EHV-1 replication was confirmed by qPCR by comparing cycle threshold (Ct) values from EHV-1 DNA extracted from filtrate prior to cell culture with DNA recovered after culture (**Table 2**). In all cases the amount of EHV-1 DNA increased, confirming CPE was caused by EHV-1 replication. Viral DNA from the samples with CPE was extracted and sequenced (PacBio RSII).

Cell culture was attempted from 12 filtered water samples from Tanzania (**Table 2**). Initially, five samples showed signs of EHV replication, with CPE visible after five days of incubation. This was confirmed by qPCR, which showed increasing GCN of EHV-1 at day 5. However, during subsequent passaging, samples were overgrown by an unknown virus.

Illumina sequencing of cDNA libraries prepared from the cultures revealed Jaagsiekte sheep retrovirus (JSRV) that was subsequently confirmed by a previously described nested PCR on water filtrate cDNA³⁹. The cell culture and water filtrates were both positive for JSRV suggesting the source of the virus was the water and not a laboratory contamination. Sanger sequencing of the PCR products showed a 99% nucleotide similarity to a previously described strain of JSRV (GenBank no NC_001494.1). No obvious reservoir for JSRV was

found in the ecosystems examined⁴⁰. We cannot exclude that an unknown reservoir shed JSRV into the water or that CPE could have derived from a different virus that we could not identify due to lack of similarity to known viruses.

pH and water temperature do not influence presence of EHV-1 in environmental water

Statistical analysis was done to determine if pH and temperature influence recovery of EHV from environmental samples. The analysis showed no significant effect of pH on EHV detection in sediment (Likelihood Ratio Test, chi-squared=1.5, p=0.22) or water (LRT, chi-squared=0.015, p=0.94). The analysis demonstrated that EHV from sediment was more likely to be detected from cooler sediment samples (LRT, chi-squared=4.7, p=0.0065; **Figure 2**). However, the statistical analysis did not reveal a significant effect of temperature on EHV detection in water (LRT, chi-squared=0.22, p=0.65; **Figure 2**). Similar temperatures were observed between sediment and water (due to the top layer of sediment being sampled, however, differences were observed between different countries sample (Supplementary table 1 and 2).

EHV host shedding during times of seasonal water shortage

Statistical analysis was performed to determine if EHV presence in fecal samples correlated with water availability, individual stress or none level, aggregation size, sex, age and reproductive status (**Supplementary table 4**). The analysis revealed that only the proxy for water availability had a significant effect on the detection of EHV in fecal samples (LRT, chi-squared=4.4, p=0.034)^{17,19}. Specifically, the model results indicated a 2.2-fold increase in the odds of detecting EHV under the dry conditions in the long grass savannah compared to the wet conditions on the short grass plains (**Supplementary figure 1**).

Phylogenetic analysis of EHV genomes isolated from water and sediment indicate limited species specificity

Of the ~150 KB EHV-1 genome, we were able to recover (PacBio) and assemble over ~130 KB from sample W20 and ~100 KB for W06 from Mongolian water filtrate (**Figure 3**). The maximum likelihood phylogenetic tree (**Figure 3**) demonstrates that the EHV-1 strains (W06 and W20) from water were most closely related to EHV-1 strain NMKT04 (KF644568). From Tanzania the water filtrate samples, three samples including two from Tanzania (PAS W051 and PAS W037) and one from Mongolia (W3) grouped within the same clade. These strains were also closely related to strains 5586 (AP012321) and Ab4p (LC193725) isolated from domestic horses (*Equus caballus*) in Japan (**Figure 3**). The most divergent strain of the alphaherpesviruses was detected from the water filtrate was sample PAS W029 which grouped closely with EHV-9 strain P19 (AP010838) isolated from a giraffe with encephalitis

(**Figure 3**). The closer phylogenetic relationship to EHV-9 isolates that are known to cross species barriers suggest these strains have the ability to infect multiple host species. The lack of diversity in strains isolated was supported by the glycoprotein B (gB), protein primase and ORF22 analysis (**Supplementary figure 2 and 3**).

Hybridization capture recovered a strain of EHV from sediment sample S3 from Mongolia. It grouped with gammaherpesviruses, although highly divergent and does not group with any known gammaherpesvirus clade (**Figure 3**). Three additional water samples from Tanzania were positive for EHV. Although full genomes could not be recovered, many of the reads obtained spanned most of the EHV genome (**Supplementary Table 3**). The results highlight the presence of both gamma and alphaherpesviruses in Mongolian samples and alphaherpesviruses in Tanzania. While most reads mapped to known EHV sequences with 100% identity, divergent reads highlight some EHV diversity in these environments (**Supplementary Table 3**).

Discussion

This study tested three main hypotheses 1) EHV hosts increase viral shedding during times of seasonal water shortage 2) once shed, EHV remain infectious in water and sediment over a prolonged period of time under natural conditions, 3) using water as an abiotic vector may restrain viral divergence. The importance of these hypotheses in supporting water as a viral vector is shown in **Figure 4**. Critically replication capable EHV was isolated from Mongolian waterholes during a time of seasonal water scarcity. Although the length of time a virus remains in individual waterholes is unknown, EHV-1 remains infectious in water for up to three weeks under laboratory conditions¹⁵. EHV-1 could not be isolated using cell culture from the EHV-1-positive African waterholes due to co-infection of the cultures with JSRV or an uncharacterized virus that was also present in the water. JSRV was absent from negative controls suggesting the culturing reagents⁴¹ were not the source. Like EHV, JSRV is an aerosol transmitted virus which may indicate water transmission is facilitated in general for viruses transmitted through this route. JSRV infects cervids⁴², however, as there are no goats or sheep in the Serengeti National Park, we can only speculate that JSRV may have originated from another cervid source. Regardless, the evidence for EHV-1 replication from Mongolian waterhole samples was unequivocal and further supported by qPCR results from African water cell culture where EHV-1 DNA levels increased over time. Despite supporting the role of water as a viral vector we cannot definitively demonstrate direct animal infection by water with our approach without employing animal infection studies. Measured abiotic factors showed little influence on EHV-1 stability in Central Asian and

East African waterholes, despite different climates. Our results suggest that viruses can survive in a diverse range of aquatic environments, pH ranges (pH 5.5-8.1) and temperatures (6.2-32.5 °C) (**Supplementary Table 1 and 2**). Despite different equid species being present in the diverse environments (**Figure 1**), stability of EHV-1 was observed, supporting previous findings that the virus is capable of remaining stable and infectious across different environmental conditions ¹⁵.

Viral water transmission may be influenced by a number of abiotic factors beyond pH and temperature. Due to the concentration effect, the number of virus particles will be inversely proportional to the water volume. Therefore, during the dry season when water volumes decrease due to evaporation, one would expect the concentration of viral particles in water would increase ^{43,44}. The wet and dry seasons will influence the number and volume of water bodies which in turn will influence the distribution of vegetation ⁴⁵. During the dry season many water bodies evaporate, vegetation concentrates around the remaining water and in turn animals become more concentrated around these limited resources. The decrease of water bodies and vegetation can be observed in Tanzania, Mongolia and Namibia during the dry season thus it is expected that animal congregations will consequently become more concentrated ^{1,2}. Thus, the concentration effect in seasonal water results in a decrease in the volume of water, increasing the concentration of virus present and an increase in aggregation of animals using the water, increasing the potential sources of the virus and transmission likelihood.

Biotic factors may also influence how viruses enter limited water sources at the most opportune times to enhance transmission. In the host species, stress would be expected to trigger viral shedding for many viruses at times of water scarcity that may result in an advantage for the virus (**Supplementary figure 1**). Physiological stress on equids such as drought and pregnancy have been linked to an increase in glucocorticoids, a measure of stress in animals ⁴⁶. Zebras located in Serengeti plains where water was collected for this study, experience increased EHV shedding from wet to dry conditions (**Supplementary figure 1**), which supports a physiological response that increases viral shedding during water scarcity. Stress may be the physiological signal that initiates reactivation of latent virus and viral shedding. Of the many factors tested, aggregation correlated with increased fecal glucocorticoid metabolite levels in Tanzanian plains zebras and, therefore, likely indicates increased stress ¹⁷. We have shown that stress may promote viral shedding in captive Grévy's zebras (*E. grevyi*) ⁴⁷. During times of water shortage, animals are forced to frequently aggregate at limited water bodies, often in mixed species assemblages ⁴⁸, and therefore the

associated stress in zebras may lead to a higher chance of viral shedding per individual⁴⁷ (**Figure 4**).

During water scarcity, animal populations are more heterogeneously distributed, spending more time confined in areas with access to water. Direct transmission among individuals may also play a role in water-borne transmission. Indeed, increased sickness may induce lethargy and increase contact rates between individuals, thus increasing viral transmission by animal-to-animal contact⁴⁹. Increased shedding in the aggregations may also increase the chance of waterborne transmission as more animals will become infected and will in turn use the limited water sources.

Viruses adapted to using water as a vector may compensate for host species heterogeneity by evolving a reduced host specificity and virulence. The absence of host specificity by several EHV species is supported by multiple lines of evidence^{50, 1,51-55}. Among Namibian wildlife, multiple non-equid species, including rhinoceros and bovines, were shown to be infected with EHV-1 and EHV-9⁵⁰. As shown in **Figure 3**, the same viral strains are found among equids and non-equids alike. Among Mongolian wildlife, there is evidence that Bactrian camels can be infected with EHV-1⁵⁶. Furthermore, Thompsons gazelles can be infected with EHV-9⁵¹. In captivity, multiple distantly related species have been shown to be susceptible to EHV-1 infection^{50,57}. Many species sympatric with equids⁵⁸, share the same waterholes and face the same drinking requirements⁵⁹, suggesting waterholes could be the source of infection for observed non-equid EHV infection. In particular, there is no evidence that shows equids interact directly with other non-equid species such that transmission would likely be indirect and mediated by abiotic vectors such as fomites or water^{60,61}.

Virulence is likely associated with virus and host co-evolutionary distance and as this distance decreases, so too should virulence. In their definitive host, EHV-1 are generally not virulent. Seroprevalence for EHV-1 can exceed 70% without increase in mortality in zebras, consistent with observations in domestic horses^{62,63}. Virulence may increase when EHV-1 and EHV-9 infect species that are not naturally sympatric, for example in zoological collections⁵⁷. Phylogenetic analysis in the current study suggests that the EHV-1 and EHV-9 are in an equilibrium state as there is only minor variation among isolates even among Western domestic horse isolates, African strains and Central Asian strains. The limited genetic variation among strains isolated in different animal species may also show that many strains may have the potential to cross species barrier such as isolated PAS-W029 which was closely related to an EHV-9 strain. This strain was isolated from a giraffe kept with Burchells's Zebras in captivity in the USA that were seropositive for the virus⁶⁴. The

absence of lineages between major EHV branches suggest that divergence and selection occurred in the past and that the sequenced EHV-1 strains have remained in equilibrium over a long period of time without generating substantial novel variation. The exception to this is that specific point mutations in the polymerase gene may influence neuropathogenesis^{23,65,66}. The conservation of the EHV-1 genome over a long evolutionary time period is consistent with reduced virulence, which may have led to a trade-off for environmental stability. The ability of the virus to remain stable and infectious in waterholes under many different conditions may also contribute to reduced host specificity for EHV-1 as more animals are likely to come into contact with the virus if it persists. Trade-offs for environmental stability are known to occur in avian influenza-A virus where trade-offs between persistence at high versus low temperatures affect the main route of viral transmission (direct or environmental) in waterfowl⁴⁴. In the case of influenza-A, this demonstrates that a trade-off for viral persistence at high temperatures leads to reduced viral persistence in the environment so direct transmission is therefore favored. However, influenza-A water vectoring may occur to a limited extent even in large water bodies⁶⁷. Water body volume was one of the crucial variables in determining the likelihood of transmission with smaller volumes increasing the likelihood. In the current study some water sources were far smaller than would be expected for waterfowl assemblages, with higher animal visitation rates and higher viral concentration which would magnify water vector transmission in such ecological contexts. Quantitation of the effects in waterholes is made difficult by the extreme variability in waterhole sizes. However, all were orders of magnitude smaller than the permanent water bodies considered for influenza-A environmental transmission^{44,68}.

Viruses in the current study were stable and infectious under different environmental conditions. Waterholes from Africa and Mongolia had high GCN concentrations of EHV as determined by qPCR. It is expected that pathogens including viruses will be diluted in larger water bodies to such a point that only those with extremely low infectious doses (influenza-A virus) will be transmissible⁶⁹. In environments that experience seasonal water shortages viruses are likely to be concentrated in waterholes during the dry season and their regular contamination is possible as increasing numbers of animals utilize the same scarce water sources. Smaller water bodies would also expose animals to larger quantities of perturbed sediment, which were shown to have higher GCN of EHV, probably due to the settling effect of the virus¹⁵. Furthermore, contamination of the water and sediment by excrement may also increase EHV viral loads¹⁹. Therefore, smaller water bodies of water are more likely to be associated EHV transmission as viruses are more likely to reach higher

concentrations increasing the chance for transmission.

In this study, we have demonstrated in two different environments and species assemblages that viruses can remain stable and infectious in waterholes through both cell culturing and molecular techniques. Viruses in addition to EHV may also be able to meet the requirements for using water as an abiotic vector (e.g., JSRV). Therefore, monitoring of waterholes for viruses may constitute a useful sentinel system for disease emergence in wildlife. Future viral evolutionary studies may need to take into account the evolutionary pressures and adaptations to water vectoring in environments that experience seasonal water scarcity.

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Competing Interests

We declare that all the authors have no competing financial interests in relation to the work described.

Data accessibility statement: The authors confirm that should the manuscript be accepted the data supporting the results will be archived in an appropriate public repository (Dryad Figshare or Hal) and the data DOI will be included at the end of the article.

Contributions of authors

AD carried out all wet lab work helped analyze data, wrote the initial manuscript, and completed revisions. PS collected sample, analyzed data and edited manuscript. AC analyzed statistical data and edited manuscript and revisions. SS collected samples. KT ran phylogenetic analysis and assembled genomes. MF analyzed statistical data and helped with revisions. GM assembled genomes. WA edited manuscript and helped with qPCR experiments. PK collected samples and edited manuscript. JM edited manuscript. ME edited manuscript. OG collected samples. CW collected samples and revised manuscript. NO revised manuscript and AD designed the study and helped write and revise the manuscript.

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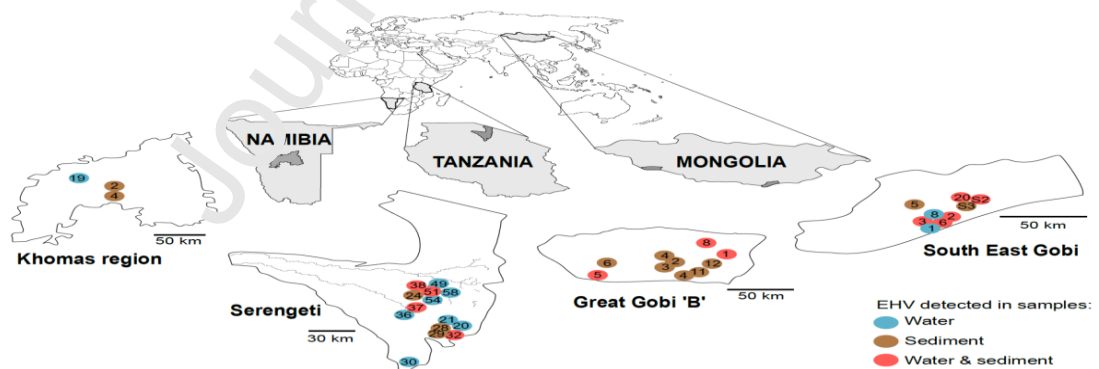


Figure 1. Map showing the sites of samples positive for equine herpesvirus (EHV) either by PCR, cell culture or qPCR. Sample collected include: sediment, water and 0.2 µl filtered water collected in Namibia, Tanzania, and Mongolia.

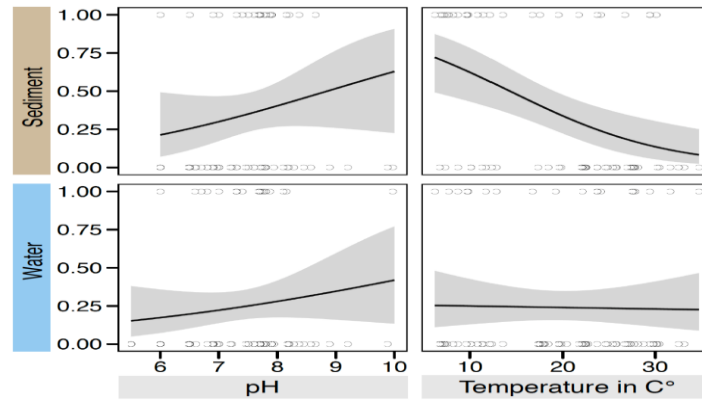


Figure 2. Detection of equine herpesvirus (EHV) in water and sediment samples in relation to temperature and pH. Sediment and water samples were collected in Namibia, Tanzania, and Mongolia. Circles represent single measurements. Lines represent predictions from the logistic regression fits. Grey areas represent 95% confidence intervals around such predictions. Predictions and confidence intervals were computed considering the mean value for the predictor not illustrated and a null random effect. Only the effect of temperature in sediment was statistically significant.

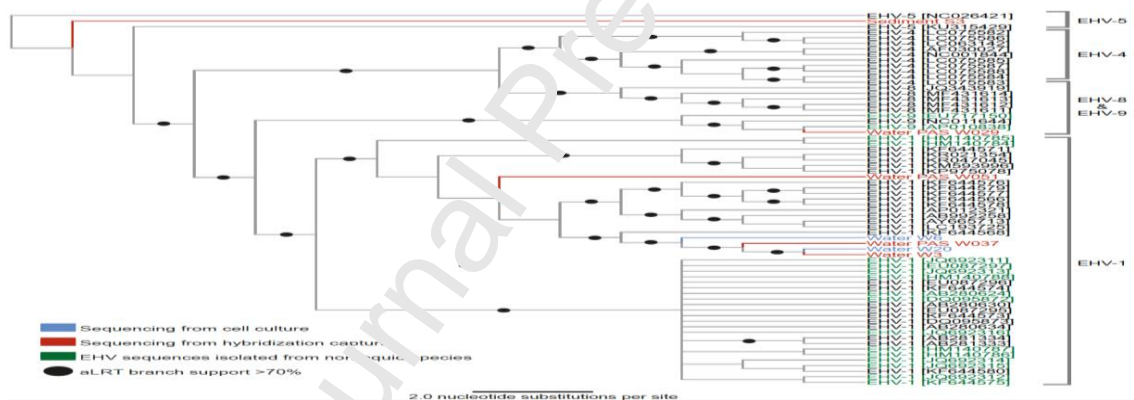


Figure 3. Maximum likelihood phylogenetic trees constructed using the JTT+Cat model. Branches are supported by approximate likelihood test ratio (aLRT). The tree shows equine herpesvirus (EHV) strains isolated from both sediment and water filtrate samples collected in Tanzania and Mongolia. Samples in blue show (near full genomes 100 KB) EHV isolates from water filtrate under cell culture conditions and subsequently sequenced using the PacBio platform. Isolates in red were recovered using hybridization capture covering varying regions of EHV genomes. Samples in green were isolated in previous studies from non-equid species.

Hybridization capture using EHV genomes as baits was performed to isolate EHV from samples where cell culture methods had failed.

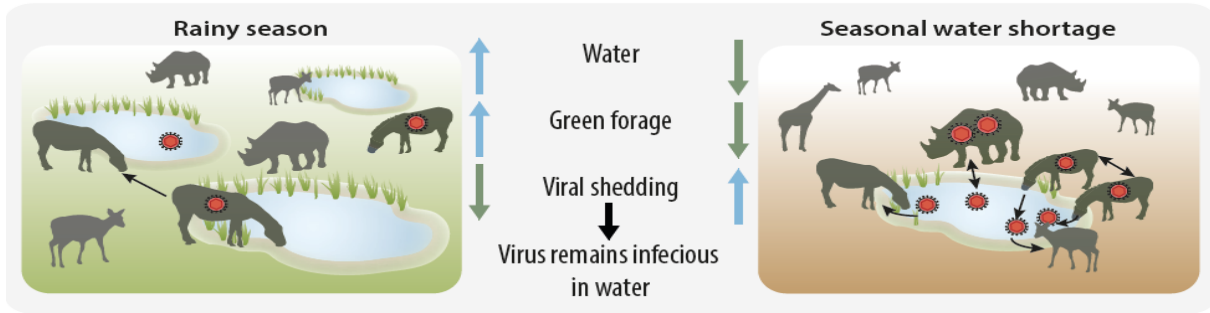


Figure 4. The difference in seasonal water availability and the factors influencing equine herpesvirus (EHV) water-borne transmission demonstrated in this study are summarized. Critical factors include, water availability and as a direct result green forage (Supplementary Figure 3), viral shedding, viral stability and viral infectivity in water. Blue arrows represent an increase in a given factor and green arrows represent a decrease.

Table 1. Number of samples (n) from both filtered water and sediment samples collected in Namibia, Tanzania and Mongolia. Equine herpesvirus (EHV) -1, -2 and -5 were detected in using both qPCR and PCR methods. The first two rows give the number of samples where different EHV strains were detected out of the total number of samples tested in PCR and qPCR. The third row also gives genome copy number (GCN) range per μL for EHV-1, -2 and -5 for all sample types collected.

Detection method	EHV strain	0.22 μL filtered water			Sediment		
		Namibia a	Tanzani a	Mongoli a	Namibi a	Tanzani a	Mongoli a
PCR (n)	EHV-1	1/32	10/34	4/25	1/20	1/44	7/25
	EHV-2	-	-	-	1/20	1/44	4/25
	EHV-5	-	-	1/25	-	-	5/25
qPCR (n)	EHV-1	1/32	8/34	7/25	1/20	5/44	9/25
	EHV-2	-	-	4/25	2/20	3/44	8/25
	EHV-5	-	-	3/25	-	-	4/25
qPCR (GCN / μL)	EHV-1	14	8-69	3-16	26	3-37	4-176
	EHV-2	-	-	18-22	38-126	25-77	17-290
	EHV-5	-	-	14-21	-	-	19-232

Table 2. Cell culture results for viral replication of equine herpesvirus (EHV) -1 cytopathic effects (CPE) after 10 days PI from 150 μL of 0.22 μm filtered water on different cell lines including rabbit kidney (RK-13), bovine kidney (MDBK) and equine dermal (ED) cells. Filtrate shows initial genome copy number (GCN) of EHV-1 of water sample. Samples sent for PacBio sequencing are indicated by grey shading.

0.22 μm filtrate	Country	Filtrate (GCN / μL)	RK-13 Cell culture (GCN / μL)	ED Cell culture (GCN / μL)	MDBK Cell culture (GCN / μL)

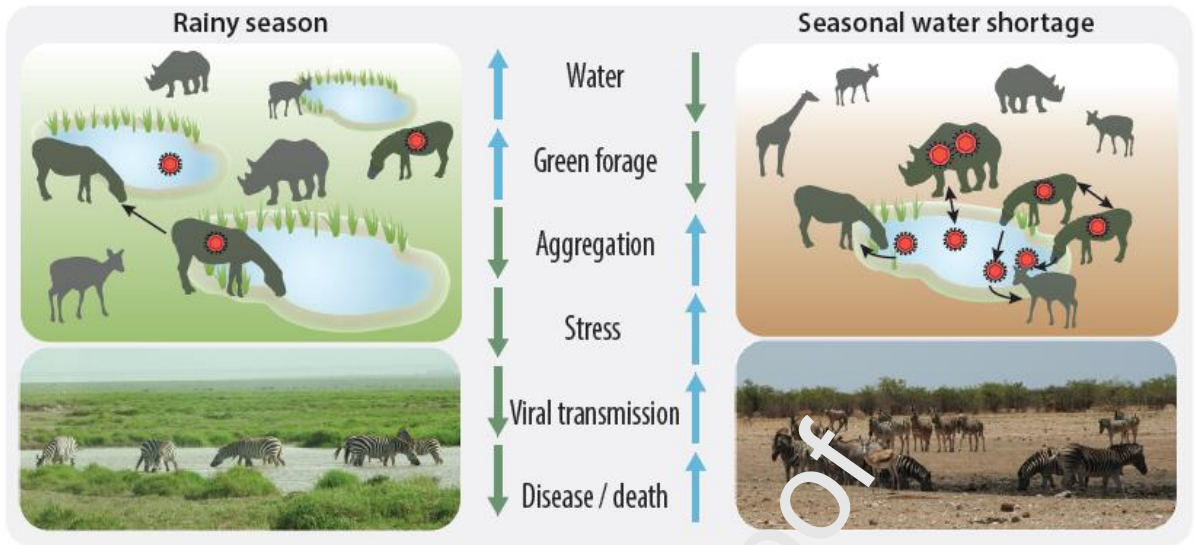
PAS-W019	Namibia	14	-	-	-
PAS-W020	Tanzania	26	-	-	-
PAS-W021	Tanzania	34	131 *	-	-
PAS-W030	Tanzania	17	-	-	-
PAS-W034	Tanzania	-	-	-	-
PAS-W036	Tanzania	-	-	-	-
PAS-W037	Tanzania	69	-	-	-
PAS-W038	Tanzania	32	86 *	-	-
PAS-W049	Tanzania	-	78 *	-	-
PAS-W051	Tanzania	19	145 *	-	-
PAS-W054	Tanzania	32	-	167	-
PAS-W058	Tanzania	15	52	-	-
S2	Mongolia	16	87	-	189 *
W3	Mongolia	14	56	-	123 *
W6	Mongolia	5	115	-	291 *
W7	Mongolia	-	-	-	-
W8	Mongolia	-	-	-	-
W20	Mongolia	11	48	-	156 *
W21	Mongolia	-	-	-	-

■ denotes samples with visible CPE after 10 days + increase in GCN / μ L

- denotes samples that were negative for CPE

* denotes samples PacBio sequenced

Graphical abstract



Highlights

- Equine herpesviruses remain infectious in environmental water
- Seasonality influences viral emergence and transmission
- Limited host specificity of Equine herpesviruses enables cross species transmission

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