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Absence of Frequent Herpesvirus Transmission in a Non-human Primate Predator-Prey System in the Wild

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Abstract

Emergence of viruses into the human population by transmission from non-human primates (NHPs) represents a serious potential threat to human health that is primarily associated with the increased bushmeat trade. Transmission of RNA viruses across primate species appears to be relatively frequent. In contrast, DNA viruses appear to be largely host specific, suggesting low transmission potential. Herein, we use a primate predator-prey system to study the risk of herpesvirus transmission between different primate species in the wild. The system was comprised of Western chimpanzees (*Pan troglodytes verus*), and their primary (Western Red Colobus; *Piliocolobus badius badius*) and secondary prey (Black-and-white Colobus; *Colobus polykomos*) monkey species. NHP species were frequently observed to be co-infected with multiple beta- and gammaherpesviruses (including new cytomegalovirus and rhadinovirus species). However, despite frequent exposure of chimpanzees to blood, organs and bones of their herpesvirus-infected monkey prey, there was no evidence for cross-species herpesvirus transmission. These findings suggest that interspecies transmission of NHP beta- and gammaherpesviruses is at most a rare event in the wild.

INTRODUCTION

Zoonotic transmission of animal pathogens into the human population is regarded as the major source of new human infectious disease (1-3). Such zoonoses have profoundly altered the course of human history, as reflected by the impact of the bubonic plague, Spanish flu and HIV/AIDS on human society (4-6). Zoonoses are frequently transmitted to humans following an initial cross-species transmission into an intermediate animal host. Mechanisms underlying cross-species transmission and adaptation to new host species are far from clear, but appear to be influenced by multiple factors, including: the level and mode of interaction between animal reservoir/transmission source and humans, the phylogenetic relationship of these species, and the nature of the zoonotic pathogen (2, 7, 8). Zoonotic/enzootic cross-species transmission appears to be a relatively common characteristic of RNA viruses (8). In contrast, the efficiency of cross-species transmission for DNA viruses is unclear. For the *Herpesviridae* family, transmission appears to be a relatively rare event. In the few instances where virus transmission has been observed, the lack of onward transmission and uncharacteristically highly pathogenic presentation of overt disease in the new species (eg., ovine/caprine herpesvirus infection in free-ranging cervids; and herpesvirus B in humans) suggest that herpesviruses poorly adapt to their new host environment (9-11).

To date, most studies examining cross-species transmission of herpesviruses have been based on phylogenetic analysis of genomic sequences. These studies reveal well-defined genotypic groupings within each of the three virus subfamilies (alpha, beta and gamma) (12). This phylogenetic distribution has been interpreted as co-evolution (co-divergence) of the major herpesvirus lineages with those of the mammalian host, with the absence of frequent cross-

70 species transmission. More recent sensitive methods of analysis using degenerate PCR targeting
71 common conserved regions of the herpesvirus genome support co-divergence as the prominent
72 mode of evolution of this virus family (13-15). However, these studies also reveal the presence
73 of repeated cross-species beta- and gammaherpesvirus transmission over evolutionary time.
74 Epstein-Barr virus (EBV) and a group of closely related African hominid gammaherpesviruses
75 (genus *Lymphocryptovirus*; LCV) were shown to be derived from at least two independent
76 introductions from Old World monkey (OWM) LCVs within the past 12 million years (14).
77 Similarly, transmission of betaherpesviruses (cytomegalovirus; CMV) was observed between
78 chimpanzees and gorillas, but the frequency of transmission, and whether transmission had
79 occurred within recent or historic time (within the last million years) could not be determined
80 (15).

81 In the present study, we use several sensitive, degenerate primer-based PCR assays for
82 the detection of herpesviruses of different genera, in combination with phylogenetic analysis
83 and specific PCR, to study cross-species beta- and gammaherpesvirus transmission in a large
84 natural primate ecosystem in the Taï National Park, Côte d'Ivoire (Western Africa). The study
85 population is comprised of a great ape predator species (Western chimpanzee; *Pan troglodytes*
86 *verus*), and its primary (Western Red Colobus, WRC; *Piliocolobus badius*) and secondary (Black-
87 and-white Colobus, BWC; *Colobus polykomos*) monkey prey, for which interspecies transmission
88 of various retroviruses has been shown (16, 17). Our study shows that each primate species is
89 infected with multiple species-specific beta- and gammaherpesviruses, but we find no evidence
90 for cross-species transmission and persistence of these viruses between the interacting ape and
91 monkey populations.

92

93 **MATERIALS AND METHODS**

94 Sample collection and DNA isolation. Necropsy samples were collected from 23 chimpanzees
95 (*Pan troglodytes verus*) (bladder, bone, brain, buffy coat, heart, heart blood, intestine, kidney,
96 liver, lung, lymph node, nasal swab, oral swab, pancreas, plasma, serum, spleen, thymus, tonsil,
97 trachea and whole blood; n=99); from 11 WRC (buffy coat, heart, kidney, liver, lung, lymph
98 node, spleen, and trachea; n=40); and from 11 BWC (buffy coat, liver, spleen, and trachea;
99 n=12) – all originating from the same area of the Taï National Park in Côte d'Ivoire. Cause of
100 death for the chimpanzees were anthrax (34), respiratory diseases (30), or undetermined (35),
101 and occurred between 2001 and 2009. The WRC and BWC were collected in the same time
102 period. For all samples originating from Côte d'Ivoire, sample collection was performed using
103 full body protection suits and masks due to a history of Ebola and Anthrax in these populations
104 and to avoid any contamination of samples with human pathogens. Permission for sample
105 collection from wild primates was obtained from the relevant authorities, and tissue samples
106 were exported with the appropriate CITES authorization from Côte d'Ivoire to Germany.
107 Sample importation adhered to German veterinary regulations for importation of organic
108 materials. All samples were preserved in liquid nitrogen upon arrival at the research camps and
109 were later transferred to -80°C at the Robert Koch-Institute. DNA was isolated using the DNeasy
110 Tissue Kit (Qiagen, Hilden, Germany).

111

112 **Herpesvirus PCR.** Details of the PCR are given below. Following PCR, all PCR products were
113 purified by using the PCR purification kit (Qiagen) and directly sequenced with a Big Dye

terminator cycle sequencing kit (Applied Biosystems, Warrington, UK) in a 377 DNA automated sequencer (Applied Biosystems).

(i) Generic CMV PCR (PCR 1 and PCR 2). For generic detection of glycoprotein B (gB; ORF UL55 in HCMV) and UL56 genes of members of the genus *Cytomegalovirus* only, gB (PCR 1) and UL56 (PCR 2), nucleic acid sequences were amplified with nested sets of degenerate primers (Table S1) derived from the gB gene and the UL56 gene of HCMV (strain AD169; accession no. NC_001347). The primer sites were located in regions conserved among the betaherpesviruses. The primers were only moderately degenerate in order to avoid amplification of roseoloviruses, alpha- and gammaherpesviruses. PCR was performed at an annealing temperature of 45^{o}C under conditions used in PCR5 (generic DPOL PCR) [below and as previously described (18)].

(ii) Long-distance (LD) PCR for amplification of WRC CMV gB sequences (PCR 3). Nested non-degenerate primers (Table S1) were designed using the sequences identified with PCR 1 and 2. Nested LD PCR of the near complete gB gene (approximately 2.2 kb) of the novel WRC CMVs was then performed using the TaKaRa-Ex PCR system according to the manufacturer's instructions (Takara Bio Inc., Japan).

(iii) Diagnostic PCR for amplification of WRC CMV gB sequences (PCR 4). For differential amplification of novel WRC CMVs, 2 specific non-degenerate primer pairs (Table S1) were designed following alignment of the 2.2 kb gB sequences obtained from the WRC CMVs. These primers were used in a nested format under the same PCR conditions as in PCR 5, except that AmpliTaq Gold was used at 0.2 µl / 25 µl reaction volume. Cycling conditions were as follows: 95^{o}C for 12 min, and 45 cycles of 95^{o}C for 30

sec, 58°C for 30 sec, and 72°C for 1 min, followed by a 10 min final extension step at 72°C. (PCR 4).

(iv) Generic herpesvirus PCR (PCR 5). For generic detection of members of the genus *Lymphocryptovirus*, sequences of the herpesvirus DNA polymerase (DPOL) gene (UL30 in HSV1; UL54 in HCMV; BALF5 in EBV; ORF9 in HHV-8) were amplified with a nested set of degenerate primers (Table S1) as described previously (18).

(v) Diagnostic PCR for amplification of WRC LCV DPOL sequences (PCR 6). For the detection of all novel WRC LCVs, specific primers (Table S1) were designed following alignment of the WRC LCV DPOL sequences identified with PCR 5. Amplification was performed under the PCR conditions of PCR 4, except that annealing was at 62 °C (PCR 6).

(vi) Generic RHV PCR (PCR 7). For generic detection of members of the genus *Rhadinovirus*, gB nucleic acid sequences were amplified with a nested set of degenerate primers (Table S1) as described previously (13).

(vii) Diagnostic PCR for amplification of WRC and BWC gB sequences (PCR 8). For the differential detection of all novel WRC and BWC RHVs, specific primers (Table S1) were deduced from an alignment of the identified DPOL sequences of WRC and BWC RHVs identified with PCR 7. They were used under the PCR conditions of PCR 4, except that annealing was at 62°C (PCR 8).

Phylogenetic analysis. Sets of nucleic acid sequences were aligned using the MAFFT [Katoh et al., 2002] plug-in of the software Geneious Pro v.5.5.7. Alignments were trimmed before using for phylogenetic analysis by removal of regions that were considered not to be justifiably

158 alignable and of loci with a gapping character in any sequence. Phylogenetic analysis was
159 performed with the Neighbor-Joining module of Geneious Pro.
160

161 **RESULTS**

162 **Detection of cytomegaloviruses in chimpanzees and colobus monkeys**

163 A core set of 130 nonhuman primate (NHP) samples in total were available for PCR-based
164 analysis. The samples consisted of tissue and blood, and nasal/oral swabs from live or deceased
165 members of the 3 primate species of the study. Samples were tested for the presence of CMVs
166 by using generic primers that detect CMVs (with the exception of roseoloviruses), alpha- and
167 gammaherpesviruses (PCR 1; Table S1). Bands of the expected product size were purified and
168 sequenced. Fifteen of 79 (19%) chimpanzee samples, corresponding to 6 of 23 individuals (26%)
169 (Table 1), were positive for CMV (PtroCMV) (Table 2). The highest percentage (31%) was found
170 in the lungs of deceased individuals (Table 2). The identified sequences originated from the
171 known chimpanzee CMVs PtroCMV1, PtroCMV2 and CCMV (Table 3; Table S2) (15). A number of
172 animals were shown to be infected with multiple CMVs (Table 4).

173 Eleven of 39 (28%) of WRC samples tested positive for CMV with the generic CMV PCR (PCR
174 1, Table S1), corresponding to 6 of 11 (54%) of animals (Table 1), with a majority of the positive
175 samples being lung and spleen samples (64%) (Table 2). Two of 12 (17%) BWC samples, both
176 derived from a single animal (Table 1), were PCR-positive for CMV (Table 2). Sequences of the
177 13 colobus-derived CMV PCR products were subjected to BLAST analysis, and determined to
178 originate from 4 formerly unknown CMV species (3 from WRC and 1 from BWC). These novel
179 CMVs were named PbadCMV1, PbadCMV1b, PbadCMV2 and CpolCMV1 (Table 3). One animal

showed the presence of multiple CMVs (Table 4). The analysis also confirmed the absence of chimpanzee CMV in any of the monkey samples.

A phylogenetic tree was constructed using a MAFFT alignment of sequences from human CMV (HCMV) (strains Toledo and AD169), great ape CMVs (chimpanzee and gorilla) and OWM CMVs (African green monkey, mandrill, rhesus macaque and multiple colobus monkey species). Inspection of the tree revealed the presence of two distinct clades: one clade was comprised of human and great ape CMVs, and the other clade of OWM CMVs. In the OWM clade, the novel WRC CMVs (PbadCMV1, PbadCMV1b, PbadCMV2) formed a distinct subclade together with a WRC CMV (designated PbadCMV3) that we had detected previously in spleens of 2 WRC (B. Ehlers, unpublished). The novel BWC CMV (CpolCMV1) was closely related to the colobus guereza virus, CgueCMV1.2 (Figure 1a).

WRC are the major monkey prey species of chimpanzees in the Taï National Park. To assess whether CMVs of WRC were present in chimpanzees, we used nested PCR primers that were designed to specifically target WRC CMVs without amplifying of chimpanzee CMV. Since the gB sequences of the novel WRC CMVs were too short for design of the necessary primers, the UL56 3'-region of 3 of the WRC CMVs (PbadCMV1, PbadCMV1b and PbadCMV2) was amplified by generic UL56 PCR (PCR 2; Table S1), followed by amplification of a 2.2kbp region between gB (UL55) and UL56 by using long-distance PCR (PCR 3; Table S1). This 2.2kbp section of PbadCMV sequence was then used for primer design (PCR 4; Table S1). Analysis using PCR 4 did not detect colobus CMV in any of the chimpanzee samples (not shown).

Detection of lymphocryptoviruses in chimpanzees and colobus monkeys

A generic herpesvirus PCR targeting the highly conserved DNA polymerase gene (DPOL) (PCR 5; Table S1) was used to analyze 39 chimpanzee samples for the presence of lymphocryptoviruses (LCV; subfamily *Gammaherpesvirinae*). Lung, spleen and lymph node samples were selected for analysis, since they had been shown to be prominent sources of gammaherpesviruses in previous studies (13, 14, 19). Sequencing of the amplified products showed 13 of 31 samples (42%) to be positive for LCV (Table 2). All sequences originated from an LCV that had 99% identity with PtroLCV1, an LCV previously identified in chimpanzees (Table 3; Table S2) (19). Spleens and lymph nodes of deceased chimpanzees showed the highest level of LCV positivity (55% and 57%, respectively). WRC (17 samples) and BWC (10 samples; including buffy coat) were analysed for the presence of LCV (PCR 5; Table S1). Five of 22 monkeys (23%) (4 WRC and 1 BWC) were positive for OWM LCV (Table 1). This was represented by six of 17 (35%) WRC samples (all from the lung and spleen), and 1 of 10 BWC samples (from buffy coat) testing positive for LCV (Table 2). BLAST analysis identified a previously reported WRC LCV (PbadLCV1) (14), and a novel BWC LCV (designated as CpolLCV1). This PCR analysis also confirmed the absence of chimpanzee LCV in the animals.

Phylogenetic analysis was performed using the corresponding DPOL region of LCV sequences from human (Epstein Barr Virus; EBV), great ape (chimpanzee and gorilla) and OWM (colobus guereza and rhesus macaque) viruses. In the tree (Figure 1b), WRC LCV (PbadLCV1 and the previously identified PbadLCV2) formed a clade distinct from a mixed clade comprising human, great ape, rhesus and BWC LCVs. Primers targeting WRC LCV (and excluding chimpanzee LCV)

were selected to test for the presence of WRC LCV in chimpanzees (PCR no. 6; Table S1). This PCR did not detect WRC LCV in any chimpanzee sample (not shown).

Detection of rhadinoviruses in chimpanzees and colobus monkeys

Gammaherpesvirus-specific gB PCR (PCR 7; Table S1) was used to analyze the 31 lung, spleen and lymph node chimpanzee samples for the presence of rhadinoviruses (RHV; subfamily *Gammaherpesvirinae*). Six of 31 samples (19%) were positive for RHV (Table 2), and the detected virus was identical to the previously identified PtroRHV2 (Table 3; Table S2) (27). Similar to the distribution of CMV, lungs of deceased chimpanzees showed a high level of RHV positivity (20%). All chimpanzee samples were also tested by using the generic DPOL PCR (PCR 5; Table S1), which resulted in detection of the known PtroRHV1 (Table 3; Table S2) (27). Similar to CMV, one animal showed the presence of multiple RHV viruses (Table 4).

In the final analysis, the 17 WRC and 10 BWC samples were tested for the presence of RHV with PCR 7. Seven of 22 (32%) monkeys (2 WRC and 5 BWC) were positive for OWM RHV (Table 1). Two of 17 WRC samples (12%) and 6 of 10 BWC samples (60%) were RHV PCR-positive, with viruses being distributed between lymphoid organs (spleen and lymph nodes), liver and blood (buffy coat) (Table 2). BLAST analysis identified the presence of two novel viruses (designated PbadRHV1 and CpolRHV1) and confirmed the absence of any chimpanzee RHV in the monkeys. All WRC and BWC samples were also tested by using the generic DPOL PCR (PCR 5; Table S1), with the same RHVs being detected (data not shown).

Phylogenetic analysis was performed using published gB sequences from human herpesvirus 8 (HHV-8), great ape RHVs (chimpanzees and gorilla), and OWM RHVs (WRC, BWC, rhesus and

pig-tailed macaque). Distinct clades of great ape RHV and OWM RHV were apparent (Figure 1c). The presence of a distinct clade containing WRC, BWC and rhesus RHV, enabled the design of clade-specific primers that detected colobus RHV, but excluded great ape RHV (PCR 8; Table S1). Use of these primers confirmed the absence of colobus RHV in all chimpanzee samples (data not shown).

Summary of DNA viruses detected and novel viruses discovered

In the present study, we detected 4 novel OWM CMVs (PbadCMV1, PbadCMV1b, PbadCMV2 and CpolCMV1), 1 novel OWM LCV (CpolLCV1) and 2 novel OWM RHVs (CpolLCV1 and PbadRHV1) in the colobus monkey study group. Together with previously identified herpesviruses detected in this study, the novel viruses are listed in Table 3 and are presented phylogenetically in Figure 1. The detection frequency of herpesviruses in individual chimpanzees was 26% (CMV), 45% (LCV) and 18% (RHV); in WRC 55% (CMV), 36% (LCV) and 18% (RHV); and in BWC 9% (CMV), 9% (LCV) and 45% (RHV) (Table 1). Individual chimpanzees and monkeys were shown to be infected by multiple herpesviruses, but with no apparent bias towards co-infection with particular viruses (Table 4). Finally, although all primate species were infected to a substantial level with their own species-specific beta- and gammaherpesviruses, there was no evidence for cross-species transmission.

DISCUSSION

We have used highly sensitive degenerate PCR in combination with specific PCR and phylogenetic analysis to analyse primate beta- (CMV) and gammaherpesviruses (LCV and RHV)

266 in a great ape predator (Western chimpanzee), and its primary (WRC) and secondary (BWC)
267 monkey prey species. Our results show that all three primate species are commonly infected
268 (and frequently co-infected) with multiple species-specific CMV, LCV and RHV herpesviruses.
269 The lung and spleen of WRC and BWC monkeys were observed to be the most frequent sites of
270 herpesvirus infection. Seven of the herpesviruses detected in this study represent new viruses
271 described for the first time.

272 Hunting frequently involves biting (both of monkeys by chimpanzees, and on occasion, of
273 chimpanzees by monkeys). Most monkey tissues, organs and bone marrow are consumed in
274 their entirety by chimpanzees. Marrow is extracted by crushing of bones, which furthers the
275 possibility for direct blood-to-blood contact by oral laceration. This predator-prey system, in
276 which chimpanzees are exposed on a continual basis to monkey blood and tissues, therefore
277 represents a unique natural primate ecosystem in which to assess microbe cross-species
278 transmission in the wild. This intensive level of interaction has been shown to lead to
279 transmission of retroviruses such as STLV-1 and SFV between chimpanzees and monkeys (16,
280 17, 20, 21). However, despite this extensive exposure, there was no evidence for cross-species
281 transmission of herpesviruses between these species.

282 Following primary infection, herpesviruses establish life-long infection within their
283 respective host species (22). Our PCR-based analysis is therefore both a measure of cross-
284 species herpesvirus transmission, and the ability of transmitted viruses to establish themselves
285 within the new host. Excluding the period of acute infection, this method of analysis will only
286 detect cross-species transmission if the virus persists following transmission, thereby avoiding
287 'background' from transient exposure to herpesviruses (such as would be detected using

288 serological-based approaches). Beta- and gammaherpesviruses are phylogenetically closely
289 grouped into distinct clades based on the specific primate species they infect. Our phylogenetic
290 analysis is therefore able to detect persistence of transmitted herpesviruses not only in
291 contemporary time, but also at the population level extending over the past 20 million years
292 (assuming an ability of transmitted viruses to be maintained within the new host species
293 population; see below). By both measures, cross-species transmission/persistence of beta- and
294 gammaherpesviruses was not detected between the different primate study populations.

295 The considerable level of interaction between prey and predator species in the primate
296 ecosystem studied here, combined with the high prevalence of herpesviruses within the two
297 species, would be expected to promote the possibility for transmission, such that exposure to
298 herpesviruses would not be the limiting factor. The absence of transmission more likely reflects
299 the inability of herpesviruses to genetically adapt to a level sufficient to infect and then persist
300 within the new primate host. Following exposure, a microbe must be able to persist and spread
301 within the new population, represented by the basic reproduction number R_0 (new infections
302 per unit time). R_0 is a critical measure of the potential for success of the pathogen within its new
303 host population, with only R_0 values > 1 being generally consistent with maintenance of an
304 enzootic/zoonotic cross-species transmission (8). Given the predator-prey nature of the
305 relationship, the possibility for transmission of microbes from chimpanzees into the monkey
306 population would be limited. However, the calculation that the average adult male chimpanzee
307 in the Taï National Forest consumes nearly 250 kilograms of colobus meat during their twenty
308 year lifetime suggests extensive exposure of chimpanzees to herpesvirus-infected monkey
309 tissue (23). Thus, the inability to detect monkey-derived herpesviruses in chimpanzees suggests

310 that primate herpesviruses maintain a high degree of species-specificity, even between related
311 primate species. It is possible that within the limits imposed by our animal group size of 24
312 chimpanzees we were unable to observe transmission/persistence events that were occurring
313 at a low frequency. Our results therefore do not rule out the possibility for herpesvirus
314 transmission between these interacting primate populations, but indicate that transmission is,
315 in the least, rare.

316 The level of genetic similarity between reservoir/transmission species and new host has
317 been suggested to play an important role in facilitating enzootic/zoonotic cross-species
318 transmission by weakening the 'species barrier', and thereby potentially increasing both I_0 (the
319 number of primary infections) and R_0. This effect of host phylogenetic similarity on transmission
320 is reflected in the high incidence of tropical zoonotic diseases that have a non-human primates
321 (NHP) source (2, 24-26). Genetic similarity between these primate species is thought to facilitate
322 pre-adaptation or rapid adaptation of the microbe, promoting its transmission and
323 establishment within humans; this can be compared to the relative inefficiency of microbes
324 moving to humans from more distantly related animal species (i.e., H5N1 avian flu from birds).
325 In the system studied here, even the presumed weak species barrier resulting from the close
326 phylogenetic relationship between the chimpanzees and their interacting monkey species
327 appears to be sufficient to prevent herpesviruses from transmitting and persisting within a new
328 primate host species.

329 RNA viruses appear to be particularly prone towards cross-species epizootic/zoonotic
330 transmission (8, 27). This propensity of RNA viruses for cross-species transmission is believed to
331 correspond to the rapid replication and high mutation rate of these viruses facilitating

adaptation to the new host environment (8, 27). In contrast, replication of DNA viruses such as herpesviruses is characterized by low level 'smouldering' or 'latent' infection with periodic reactivation (increased levels of herpesvirus replication and overt disease during the chronic phase of infection are generally seen only associated with immunosuppression). DNA viruses also have far higher fidelity of replication than observed for RNA viruses (27). Both of these factors may result in reducing the potential for adaptation of herpesviruses to a new host, negatively impacting I_0 and R_0, and reducing capacity for cross-species epizootic/zoonotic transmission.

Our current study would suggest that relatively strict species specificity exists for primate beta- and gammaherpesviruses. Previous results from *in vitro* studies are consistent with our findings, with species-specific CMVs replicating poorly in cells from other species (28-30). In these studies, the genetic similarity between host species appeared to influence the replicative capacity of the respective CMVs, with HCMV replication being reduced only 10-fold in chimpanzee cells, compared to being non-detectable in cells from mice. *In vivo* studies support this level of species specificity, with no cross-species transmission/persistence being observed for murine CMV (MCMV) from naturally infected *M. domesticus* (house mouse) to native *L. lakedownensis* (short-tailed mice) following the release of MCMV-infected house mice into the Thevenard Island natural reserve (31). In the Thevenard Island study, MCMV did not replicate even following direct inoculation of virus into *L. lakedownensis*.

The capacity for transmission of gammaherpesviruses has not been empirically examined. However, degenerate PCR-based approaches, similar to those used in the present study, indicate that cross-species transmission/persistence has occurred for both beta- and

gammaherpesviruses at least on an evolutionary time-scale – although the scarcity of these events would support that such transmission is rare. Specifically, phylogenetic analysis provides evidence for transmission of CMV between ape species (chimpanzees and gorillas) within the last million years, and of at least two independent introductions of OWM LCV into ape populations around 12 million years ago (14, 15). An OWM LCV transmission into Asian apes (orang-utans and gibbons) is also believed to have occurred more recently, approximately 1 million years ago (14). There is also evidence for transmission of non-primate herpesviruses (specifically, RHVs) (13). Interestingly, the close phylogenetic relationship of RHVs from spotted hyena with those of zebra/horses, and of lion RHV with those of wild pig/rhino species, suggest that a predator and prey interaction may be one scenario that favours cross-species herpesvirus transmission. Together with results from these earlier studies, the lack of evidence for transmission/persistence of primate beta- and gammaherpesviruses in our current study suggests that although viruses from these two herpesvirus families are capable of cross-species transmission/persistence, such events are rare on both a contemporary and evolutionary time-scale.

Due to high immunogenicity and 'effector' T cell memory bias of CMV-induced immune responses, a number of laboratories are developing CMV as a vaccine platform (32-36). CMVs have evolved to spread through their target host population, and have a remarkable capacity to reinfect the host regardless of prior CMV immunity (37). We and others are therefore beginning to exploit this ability of CMV to spread for the development of 'disseminating' vaccines to target animal populations that are geographically or economically inaccessible to standard vaccination strategies (for example, to prevent Ebola virus infection in great apes in Central Africa, or as an

immunocontraception in mice to prevent mouse plagues) (33, 35). The present study furthers our understanding of the capacity for cross-species transmission of CMV between closely related species in a natural ecosystem, which will be critical as these vaccine strategies move towards potential application.

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Figure 1. Phylogenetic analysis. Partial gene sequences from the herpesviruses detected in this study and from published herpesviruses were aligned using MAFFT and subjected to phylogenetic construction of trees using the Geneious 5.5.7 tree builder (Neighbor-Joining module). Human, great ape and OWM herpesviruses are in red, green and blue font, respectively. Viruses detected in this study are marked (black dot). (A) tree based on glycoprotein B sequences of CMVs; (B) tree based on DNA polymerase sequences of LCVs; (C) tree based on glycoprotein B sequences of RHVs. Bootstrap values are indicated at the basis of major clades and suppressed at the tips of the clades.

Table 1 NHP individuals positive in generic PCR

	Chimpanzee	WRC	BWC
Cytomegaloviruses	23 (6) [a]	11 (6)	11(1)
Lymphocryptoviruses	22 (10)	11 (4)	11(1)
Rhadinoviruses	22 (4)	11 (2)	11(5)

[a] number of individuals tested (number of individuals positive in generic PCR)

514 **Table 2 Organs of NHP positive in generic PCR**
515

	Chimpanzee	WRC	BWC
Cytomegaloviruses			
Lung	16 (5)[a]	8 (4)	-
Spleen	7 (1)	7 (3)	1 (1)
Liver	10 (2)	7 (2)	1 (1)
Heart	5 (1)	2 (1)	-
Lymph node	11 (1)	2	-
Intestine	12	-	
Tonsil	6 (2)	-	-
Kidney	3	2 (1)	-
Whole blood	4 (1)	-	-
Thymus	1 (1)	-	-
Pancreas	1 (1)	-	-
Brain	2	-	-
Bladder	1	2	-
Trachea	-	-	1
Blood (buffy coat)	-	8	9
Heart blood	-	1	-
Sum	**79 (15)**	**39 (11)**	**12 (2)**
Lymphocryptoviruses			
Lung	15 (4)	8 (3)	-
Spleen	7 (4)	7 (3)	1
Lymph node	9 (5)	2	-
Liver	n.d.	n.d.	1
Trachea	-	-	1
Blood (buffy coat)	-	n.d.	7 (1)
Sum	**31 (13)**	**17 (6)**	**10 (1)**
Rhadinoviruses			
Lung	15 (3)	8	-
Spleen	7 (1)	7 (1)	1 (1)
Lymph node	9 (2)	2 (1)	-
Liver	n.d.	n.d.	1 (1)
Trachea	-	-	1
Blood (buffy coat)	-	n.d.	7 (4)
Sum	**31 (6)**	**17 (2)**	**10(6)**

516
517 [a] number of samples (number of samples PCR-positive in generic PCR)
518 - = not available; n.d. = not done

519
520
521 **Table 3 Herpesviruses detected in chimpanzees, WRC and BWC by generic PCR**
522

Host species	Virus	Abbreviation	Virus novel or known	Generic PCR[a]	No. of PCR-positive animals	No. of PCR-positive samples
Catarrhini						
Family: Hominidae						
Western chimpanzee	CCMV	CCMV	k	gB	2	3
	Pan troglodytes verus cytomegalovirus 1	PtroCMV1	k	gB	2	5
	Pan troglodytes verus cytomegalovirus 2	PtroCMV2	k	gB	4	7
	Pan troglodytes verus lymphocryptovirus 1	PtroLCV1	k	DPOL	10	14
	Pan troglodytes verus rhadinovirus 1	PtroRHV1	k	DPOL	4	6
	Pan troglodytes verus rhadinovirus 2	PtroRHV2	k	gB	1	1
Family: Cercopithecidae						
Western red colobus	Piliocolobus badius cytomegalovirus 1	PbadCMV1	n	gB	1	4
	Piliocolobus badius cytomegalovirus 1b	PbadCMV1b	n	gB	2	2
	Piliocolobus badius cytomegalovirus 2	PbadCMV2	n	gB	3	6
	Piliocolobus badius lymphocryptovirus 1	PbadLCV1	k	DPOL	4	5
	Piliocolobus badius rhadinovirus 1	PbadRHV1	n	gB	2	3
Black-and-white colobus	Colobus polykomos cytomegalovirus 1	CpolCMV1	n	gB	1	2
	Colobus polykomos lymphocryptovirus 1	CpolLCV1	n	DPOL	1	1
	Colobus polykomos rhadinovirus 1	CpolRHV1	n	gB	5	6

523
[a] k, known; n, novel
[a] Target genes are listed: gB, glycoprotein B; DPOL, DNA polymerase
524
525

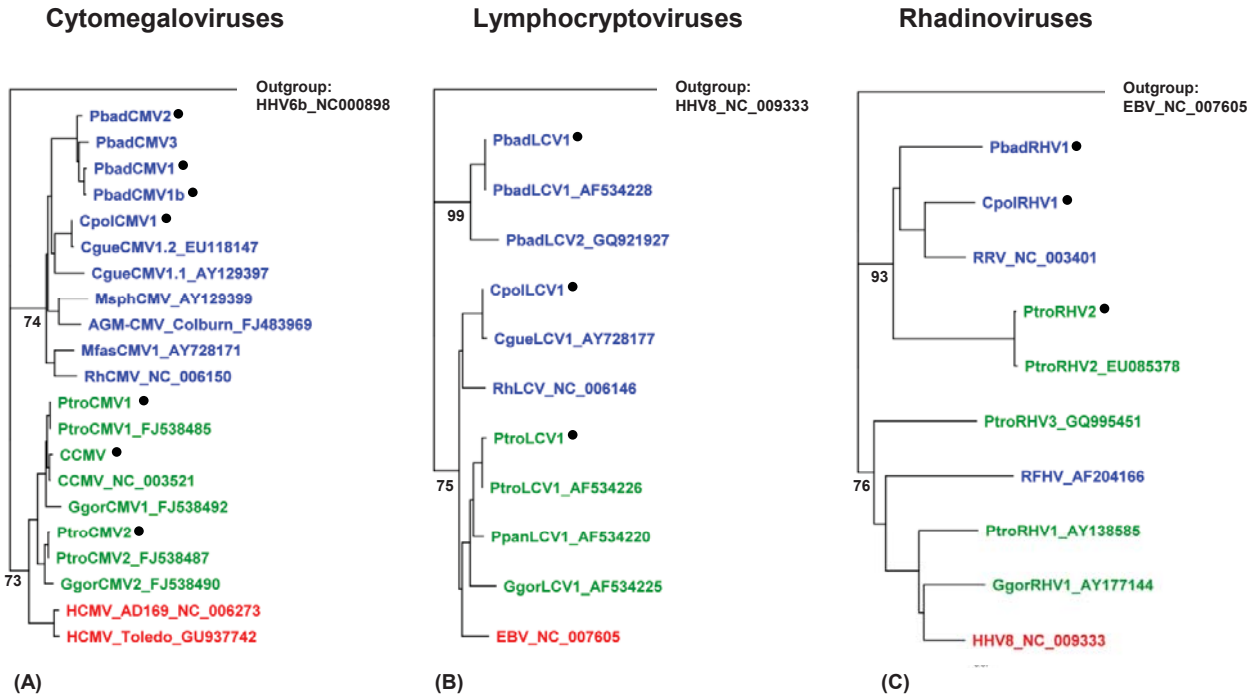
526

527 **Table 4 Coinfections**

Individual	Cause of death	Generic PCR-positive	Cytomegalovirus	Lymphocryptovirus	Rhadinovirus
Chimpanzee					
no. 2 “Leo”	Anthrax	Lung, lymph node, spleen	PtroCMV2	-	PtroRHV1; PtroRHV2
no. 10 “Noah”	Anthrax	Heart, liver, lung, pancreas, thymus	PtroCMV2, PtroCMV1, CCMV	PtroLCV1	-
no. 21 “Ophelia”	Respiratory disease	Liver, lung, spleen, tonsils	PtroCMV1	PtroLCV1	-
no. 76 “Candy”	Respiratory disease	Lung and spleen	-	PtroLCV1	-
no. 560 “Akwaba”	Respiratory disease	Lung and tonsils	PtroCMV1 PtroCMV2	PtroLCV1	-
Colobus					
no. 66 (WRC)	Undetermined	Lung	PbadCMV1b	-	PbadRHV1
no. 71 (WRC)	Undetermined	Lung	PbadCMV1b, PbadCMV2	PbadLCV1	-
no. 72 (WRC)	Undetermined	Spleen	PbadCMV2	-	PbadRHV1
no. 213 (WRC)	Undetermined	Spleen	PbadCMV2	PbadLCV1	-
no. 740 (BWC)	Undetermined	Slpeen,trachea	CpolCMV1	-	CpolRHV1

528 -, PCR negative

529


Cytomegaloviruses
Lymphocryptoviruses
Rhadinoviruses
Outgroup:
HHV6b_NC000898
PbadCMV2
PbadCMV3
PbadCMV1
PbadCMV1b
CpolCMV1
CgueCMV1.2_EU118147
CgueCMV1.1_AY129397
MsphCMV_AY129399
AGM-CMV_Colburn_FJ483969
MfasCMV1_AY728171
RhCMV_NC_006150
PtroCMV1
PtroCMV1_FJ538485
CCMV
CCMV_NC_003521
GgorCMV1_FJ538492
PtroCMV2
PtroCMV2_FJ538487
GgorCMV2_FJ538490
HCMV_AD169_NC_006273
HCMV_Toledo_GU937742
74
73
(A)
Outgroup:
HHV8_NC_009333
PbadLCV1
PbadLCV1_AF534228
PbadLCV2_GQ921927
CpolLCV1
CgueLCV1_AY728177
RhLCV_NC_006146
PtroLCV1
PtroLCV1_AF534226
PpanLCV1_AF534220
GgorLCV1_AF534225
EBV_NC_007605
99
75
(B)
Outgroup:
EBV_NC_007605
PbadRHV1
CpolRHV1
RRV_NC_003401
PtroRHV2
PtroRHV2_EU085378
PtroRHV3_GQ995451
RFHV_AF204166
PtroRHV1_AY138585
GgorRHV1_AY177144
HHV8_NC_009333
93
76
(C)