ORIGINAL ARTICLE

A novel herpesvirus in the sanctuary chimpanzees on Ngamba Island in Uganda

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Abstract

Background Recent studies in non-human primates have led to the discovery of novel primate herpesviruses. In order to get more information on herpesvirus infections in apes, we studied wild born captive chimpanzees.

Methods Chimpanzees of the Ngamba island sanctuary, Uganda, were analyzed with pan-herpes polymerase chain reaction (PCR) targeting the herpesvirus DNA polymerase gene and the glycoprotein B gene. The obtained sequences were connected by long-distance PCR, and analyzed phylogenetically.

Results Twenty-one of 40 individuals were infected with members of the *Gammaherpesvirinae*, two of them with a novel member of this subfamily. Phylogenetically, the novel virus fell into a clade of primate rhadinoviruses and the Kaposi sarcoma herpesvirus (human herpesvirus 8), representing a third distinct rhadinovirus in chimpanzees.

Conclusion Non-human primates harbor several herpesviruses many of which are still unknown. This has implications to management of primates in sanctuaries requiring continuous updates on the management protocols to deal with potential occupational pathogens.

Introduction

Little is known about the spectrum of infectious agents carried by wild non-human primates. However, such baseline data would be helpful to identify potential zoonotic threats and to better understand players involved in diseases threatening the health of non-human primates [11, 20]. Herpesviruses may play an important role in multifactorial disease progressions, and despite various discoveries of novel herpesviruses in wild great apes and other primates [8, 18, 20], the spectrum of herpesviruses is far from being described.

The members of the family *Herpesviridae* have been previously grouped into three subfamilies, designated *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* [28], and phylogenetic lineages in these subfamilies were identified [22–25]. In studies amplifying unknown herpes virus sequences from the DNA polymerase (DPOL) gene or – more recently – from two conserved genes by a bigenic polymerase chain reaction (PCR) approach, novel herpesviruses were continuously discovered. For example, 14 novel gammaherpesviruses (GHV) were discovered in eight different host species from six mammalian orders [8], and 10 novel betaherpesviruses were identified in great apes [19]. These discoveries were based on sequences encompassing two or more conserved genes, and determined from long-distance (LD)-PCR products [8, 9, 19, 27].

Herpesviruses are widespread in vertebrate species, sharing several moderately to well-conserved genes, as determined from amino acid identity comparisons (e.g. DNA polymerase and glycoprotein B). Within

the GHV, Epstein–Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV), classified as *Human herpesvirus 4* (HHV-4) and *Human herpesvirus 8* (HHV-8), are the human members of the *Lymphocryptovirus* genus and the *Rhadinovirus* genus respectively. Both of these viruses play a critical role in human multistep carcinogenesis, especially in immunodeficient patients, leading to Burkitt's lymphoma [21] and Kaposi's sarcoma [5, 31] respectively. GHV of the genus *Rhadinovirus* have also been detected in several mammalian species including New World monkeys (squirrel monkey, spider monkey) [1, 2] and Old World monkeys (several macaque species, African green monkey, mandrill) [3, 7, 13, 15, 29, 32]. Comparison and phylogenetic analyses of available sequences support the existence of two distinct lineages among the Old World monkey rhadinoviruses, called RV1 and RV2 for the *Rhadinovirus* lineages 1 and 2 [4, 13, 15, 30]. KSHV belongs to the RV1 lineage, whereas no human virus has yet been discovered in the RV2 lineage.

Previous studies on African great apes from Cameroon and Gabon have shown that they harbor different rhadinoviruses closely related to KSHV. By serology and PCR, common chimpanzees were determined to be infected with rhadinoviruses of the RV1 and the RV2 lineage being termed Pan Rhadino-herpesvirus 1a, 1b, 2 (PanRHV1a, PanRHV1b, PanRHV2) [16, 17]. Using a bigenic LD-PCR approach, rhadinoviruses very similar to PanRHV1a and PanRHV2 were found in chimpanzees from Ivory Coast (PtroRHV-1 and PtroRHV-2) [27]. These studies demonstrate that wild animals, especially primates, harbor many unknown viruses, and further search will continue to reveal novel pathogens.

In this study, we analyzed wild born captive chimpanzees residing at Ngamba Sanctuary in Uganda to gain more insights into the herpesviruses harbored by this community. The results would fit into improvements of management protocols including employee health programs and future plans for re-introductions.

Materials and methods

The study was conducted on 40 orphan chimpanzees (*Pan troglodytes schweinfurthii*) in semi-captive management at Ngamba Island Chimpanzee Sanctuary, Uganda. The total group consists of 23 females and 19 males with age group categorized as follows: 4 infants (1–5 years), 6 juveniles (6–8 years), 12 subadults (9–11 years) and adults (12 years and more). These chimpanzees were brought to the sanctuary at different times after being rescued from illegal traders and poachers mainly from Uganda and Democratic Republic of Congo since 1998 when the sanctuary started.

Blood was collected from 40 chimpanzees under general anesthesia during our annual medical health checks in February 2007. Whole blood (10 ml) was taken by inguinal venipuncture using CPT Vacutainer tubes. Leucocytes/cells from fresh whole blood collected in CPT tubes were processed using a cell preparation procedure within 12 hours. The processed cells were stored in cryotubes under liquid nitrogen (−196°C) and stored at Uganda Virus Research Institute till transported on dry ice to Robert Koch Institute for analysis.

The research was approved by Uganda Wildlife Authority and Uganda National Council of Science and Technology. In addition to international requirements, the local and regional requirements of managing chimpanzees in sanctuaries established by International Primatological Society and Pan African Sanctuaries Alliance were followed as part of routine management of chimpanzees.

DNA extraction, amplification, purification, cloning and sequencing

DNA was extracted from buffy coat of the whole blood cells of 40 chimpanzees using QIAamp DNA Blood Min Kit (QIAGEN®, QIAamp®, Hilden, Germany) according to manufacturer's instructions.

Panherpesvirus consensus PCR for the amplification of 160–181 bp (excluding primer-binding sites) of the DPOL gene was carried out as described previously [6]. Samples that did not yield an amplification product were rerun under more relaxed conditions; i.e. the ramp time between the annealing step and the extension step was prolonged 50-fold, and the final concentration of the polymerase was increased twofold.

For the amplification of the gB genes of as yet unknown GHVs, two degenerate, deoxyinosine-containing primer sets (GH1 and GH2) were used [8].

Nested LD-PCR was performed with the TaKaRa-Ex PCR system according to the instructions of the manufacturer (Takara Bio Inc., Otsu, Japan) by using virus-specific primers (not listed) as described previously [8, 12]. PCR products were purified by using the PCR purification kit (QIAGEN) and directly sequenced with the Big Dye terminator cycle sequencing kit (Applied Biosystems, Warrington, UK) in a 377 DNA-automated sequencer (Applied Biosystems).

Multiple sequence alignment and phylogenetic analysis

Analysis was carried out at the level of encoded amino acid sequences. Sets of partial amino acid sequences from DPOL and gB were aligned using CLUSTALW [33].

Positions in alignments that had missing characters in any sequence were removed before the alignment was used for phylogenetic analyses with the neighbor-joining method as performed previously [27].

Nucleotide sequence accession numbers

The following DPOL and gB sequences of known primate herpesviruses were used for comparative purposes: EBV = HHV-4, GenBank accession number (acc.-no.) NC_009334; Pan troglodytes lymphocryptovirus (PtroLCV-1), herpesvirus pan = *Panine herpesvirus 1* (PnHV-1), acc.-no. AF534226; Rhesus lymphocryptovirus (RhLCV) = *Macacine herpesvirus 4* (McHV-4), acc.-no. NC_006146; Marmoset lymphocryptovirus (MaLCV) = Callitrichine herpesvirus 3 (CalHV-3), acc.-no. NC_004367; KSHV = HHV-8, acc.-no. NC_003409; Rhesus rhadinovirus (RRV) = *Macacine herpesvirus 5* (McHV-5), acc.-no. NC_003401; Pan troglodytes rhadinovirus 1 (PtroRHV-1), acc.-no. AY138585; Pan troglodytes rhadinovirus 2 (PtroRHV2), acc.-no. AY138585; Macaca fascicularis rhadinovirus 1 (MfasRHV-1), acc.-no. AY138583; Macaca fascicularis rhadinovirus 2 (MfasRHV2), acc.-no. EU085377; Retroperitoneal fibromatosis-associated herpesvirus (RFHV), acc.-no. AF204166; Herpesvirus Saimiri (HVS) = *Saimiriine herpesvirus 2* (SaHV-2), acc.-no. NC_001350; Chimpanzee cytomegalovirus (CCMV) = *Panine herpesvirus 2*, acc.-no. AF480884. The nucleotide sequence of PtroRHV-3 was deposited in GenBank under the acc.-no. GQ995451).

Results and discussion

Twenty-one of 40 samples from chimpanzees (*Pan troglodytes schweinfurthii*) analyzed with pan-herpes DPOL PCR were tested positive for GHV. The sequences from five samples originated from PtroRHV-1 (acc.-no. AY138585) [27]. Fourteen samples were positive for the lymphocryptovirus PtroLCV-1 (acc.-no. AF534226) [10]. Two cases appeared to harbor a novel gammaherpesvirus, which was tentatively named *Pan troglodytes* rhadinovirus 3 (PtroRHV-3). All results are listed Table 1.

With degenerate primers targeting the gB gene, a 453-bp sequence was amplified from one of the PtroRHV-3-positive samples. From the partial gB and DPOL sequences of PtroRHV-3, specific nested primers were deduced and used in LD-PCR. The 3.3-kb product was sequenced and a final contiguous PtroRHV3 sequence was compiled spanning 3446 bp.

In a pair-wise nucleic acid sequence comparison, the 3.4-kbp sequence of PtroRHV-3 was found to be 69%

identical to PtroRHV-1. The 3′end of the PtroRHV-3 sequence (0.4 kb) was 99% identical to a rhadinovirus (PanRHV1b) found in *Pan troglodytes troglodytes* [17]. No close matches were found in GenBank for the 5′-part (3 kbp) of the PtroRHV-3 sequence, spanning a part of the DPOL and the gB gene. As the PtroRHV-3 sequence was detected in *Pan troglodytes schweinfurthii* from Uganda and the Democratic Republic of Congo, it was regarded as originating from a hitherto unknown *P. t. schweinfurthii* RHV, closely related to *P. t. troglodytes* RHV1b.

From the partial gB and DPOL open reading frames (1020 and 2376 bp respectively), amino acid sequences were deduced, concatenated and aligned with corresponding aa primate GHV sequences from GenBank. Phylogenetic tree analysis was performed as described previously [27], and aa sequences of the chimpanzee cytomegalovirus (GenBank acc.-no. AF480884) were used for rooting. In the tree, the novel virus clustered more closely with the human rhadinovirus (KSHV/HHV-8) and with PtroRHV-1 than with PtroRHV2 (Fig. 1).

This study confirms that there are three distinct rhadinoviruses within the *Gammaherpesvirinae* of chimpanzees [16]. Although the human rhadinovirus HHV-8 is closely related to PtroRHV-1 (and PanRHV1a), no human rhadinoviruses with close similarity to PtroRHV-2 or PtroRHV-3 are known. Nevertheless, they could exist, as herpesviruses co-evolve with their hosts.

The human gammaherpesviruses EBV and KSHV pose significant health risks worldwide, including the induction of cancer and lymphoproliferative diseases. They pose a risk of serious disease especially to those lacking a competent immune system, such as neonates, transplant patients and people suffering from AIDS (reviewed in Ref. [26]). Although acute gammaherpesvirus infection is rapidly cleared by a strong immune response, the establishment of latent infection in hematopoietic cells allows these viruses to successfully evade the host immune response and maintain lifelong infection. To date, the pathogenesis and immunity of gammaherpesvirus infections are still not well understood and therefore convincing strategies to prevent long-term latency have not been realized [35].

It has been demonstrated in studies with mice that the level of latent infection with a gammaherpesvirus is independent of the infectious dose over at least a 104-fold range, regardless of whether inoculation occurs via a systemic or a mucosal route [34]. Host factors like a compromised immune system have been implicated in the initiation of infection leading to substantial acute phase replication and subsequent establishment of a

**Table 1** Chimpanzee biodata and gammaherpesviruses detected

| Chimpanzee biodata | | | | Gammaherpesvirinae | | |
| | | | | Lymphocryptovirus | Rhadinovirus | |
Name of chimpanzee	Sex	Age	Origin	PtroLCV-1	PtroRHV-1	PtroRHV-3
Kidogo	F	23	DRC	–	–	–
Sophie	F	21	DRC	+	–	–
Katie	F	20	DRC	+	–	–
Ikuru	F	12	DRC	+	–	–
Billi	F	10	DRC	+	–	–
Mukisa	F	9	DRC	–	–	+
Ndyakira	F	8	DRC	–	–	–
Nakuu	F	6	DRC	+	–	–
Masiko	M	23	DRC	–	–	–
Sunday	M	20	DRC	+	–	–
Robbie	M	21	DRC	–	–	–
Mawa	M	11	DRC	–	–	–
Kalema	M	11	DRC	–	–	–
Umutama	M	11	DRC	–	–	–
Umugezi	M	10	DRC	+	–	–
Baluku	M	9	DRC	+	–	–
Asega	M	9	DRC	–	–	–
Kisembo	M	8	DRC	+	–	–
Indi	M	8	DRC	+	–	–
Okech	M	8	DRC	–	+	–
Rambo	M	3	DRC	–	+	–
Megan	F	23	Rwanda	–	–	–
Kyewunyo	F	5	Uganda	–	+	–
Peace	F	21	Uganda	–	–	–
Connie	F	18	Uganda	–	–	–
Bahati	F	17	Uganda	–	+	–
Natasha	F	17	Uganda	–	–	–
Becky	F	16	Uganda	–	–	–
Sally	F	16	Uganda	–	–	–
Cindy	F	14	Uganda	–	–	–
Nkumwa	F	11	Uganda	+	–	–
Kazahukire	F	8	Uganda	–	–	–
Tumbo	M	18	Uganda	–	+	–
Mika	M	15	Uganda	–	–	–
Bwambale	M	7	Uganda	–	–	+
Rutoto	M	3	Uganda	+	–	–
Nagoti	F	21	Unknown	+	–	–
Nani	F	6	Unknown	+	–	–
Sum of PCR-positive samples				14	5	2

DRC, Democratic Republic of Congo.

maximal level of latency. In addition, it is not known whether the gammaherpesviruses found in great apes present the same way as their human counterparts both in establishment of infection and pathogenicity. For example, it has now been shown that chimpanzees harboring simian immunodeficiency virus have a higher likelihood of dying and lower reproductive rate [14]. Most likely, the underlying pathological processes are multifactorial, and herpesviruses may play an important

role. Therefore, detailed analysis in long-term studies is needed.

Finally, not only co-evolvement with the host but also horizontal transmission appears to be an important component of beta- and gammaherpesvirus evolution [8, 19; B. Ehlers and D. McGeoch, unpublished data]. These circumstances and uncertainties present challenges in the management of occupational hazards in captive ape facilities (sanctuaries and zoos)
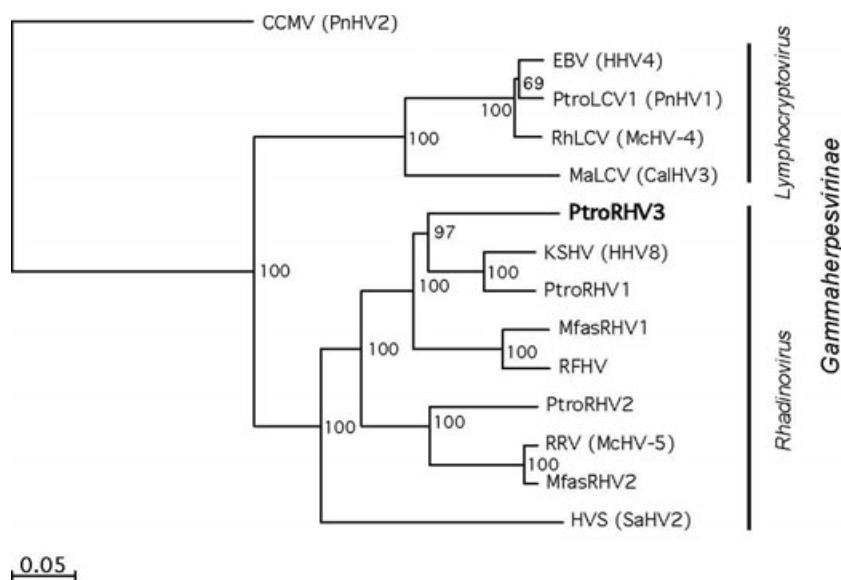
Fig. 1 Phylogenetic analysis of the novel chimpanzee rhadinovirus. A phylogenetic tree was constructed using the amino acid (aa) sequences encoded by the gB-DPOL segments of the novel primate herpesviruses and of known human and non-human primate herpesviruses, available in GenBank. Abbreviations of common names are used, and those of species names according to the ICTV (International Committee on the Taxonomy of Viruses) are given in parentheses. A multiple alignment of concatenated 1100 aa was analyzed with the neighbor-joining method. A rooted phylogram is shown, with CCMV as outgroup. The branch length is proportional to evolutionary distance (scale bar). Results of bootstrap analysis (1000 replicates) are indicated at the nodes of the tree. The novel PtroRHV-3 is highlighted in bold type. Full names of known viruses and their nucleotide sequence accession numbers are listed in the Materials and methods section.

where there is close interaction of employees with the apes.

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