

## **Partial molecular characterization of alphaherpesviruses isolated from tropical bats**

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Herpesviruses have previously been isolated from African and South-American bats. Recently, herpesviruses detected from European insectivorous bats (family Vespertilionidae) were classified molecularly as betaherpesviruses and gammaherpesviruses. In the current study, we performed PCR analyses targeting the UL30 catalytic subunit region of the DNA polymerase gene of the African and South American herpesviruses and new Malagasy and Cambodian herpesviruses isolated from bats, especially frugivorous bats from the families Pteropodidae and Phyllostomidae. The sequences obtained from the amplified products indicated that these isolates belonged to the genus *Simplexvirus* of the subfamily *Alphaherpesvirinae*. These results extend the taxonomic range of bat herpesviruses with the description of four members in the subfamily *Alphaherpesvirinae*. Furthermore, these data confirm and extend the geographical distribution of herpesvirus in bats to three more continents (Africa, South America and Asia) and indicate the presence of these viruses in frugivorous bats of the families Pteropodidae and Phyllostomidae.

The family *Herpesviridae* includes about 124 virus species (Büchen-Osmond, 2001). The diagnostic characters of this family are based on virus structure (large, enveloped virus, from 120 to 200 nm, icosahedral capsid, large double-stranded DNA of 120000–220000 bp). Viruses of this family exhibit considerable differences in their nucleotide composition and genome organization. However, they share several biological properties including their ability to be latent or persistent for life in their host. On the basis of differences in cellular tropism, genome organization and gene content, the family *Herpesviridae* has been divided into three subfamilies, *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*. Each subfamily includes genera and species grouped together based on, for example, DNA polymerase sequences and similarities in genome sequence arrangement (Roizman, 1996; McGeoch *et al.*, 2006).

Herpesviruses are highly disseminated in vertebrates, and most mammal orders have yielded at least one herpesvirus. No herpesvirus is listed in the universal virus database from the order Chiroptera or bats (Büchen-Osmond, 2001). However, bats are not an exception. Herpesviruses, among numerous viruses from different families, have been obtained from bats, including a cytomegalovirus isolated from an insectivorous bat (*Myotis lucifugus*, family Vespertilionidae) in the USA and Agua preta and Parixa viruses isolated from frugivorous bats (family Phyllostomidae) in Brazil (Calisher *et al.*, 2006). Further isolates have been obtained from frugivorous bats (family Pteropodidae) in Cameroon (Dak An Y 6, Dak An Y 7 and Dak An Y 9) and from an unidentified bat species in the Central African Republic (Dak An B N27)

(<http://www.pasteur.fr/recherche/banques/CRORA/virus/v1301010.htm>). Allocation to the family *Herpesviridae* was based on serological tests or electron microscopy; however, these viruses were not studied in a molecular manner and have not been assigned to a genus in the family. Recently, herpesvirus sequences targeting the DNA polymerase gene were detected from European insectivorous bats (family Vespertilionidae) and related to members of the gammaherpesvirus genera *Percavirus*, *Rhadinovirus* and *Macavirus* and of the subfamily *Betaherpesvirinae* (Wibbelt *et al.*, 2007).

In the present study, we report on the partial molecular characterization of Parixa (Be AN 422840), Dak An Y 6, Dak An Y 7, Dak An Y 9 and Dak An B N27 herpesviruses isolates and 10 additional herpesvirus isolates that we obtained recently from Malagasy and Cambodian frugivorous bats, in order to confirm their allocation to the family *Herpesviridae* and to assign a genus name to these isolates.

Fifteen isolates were analysed, including 10 undescribed isolates obtained on Vero E6 cells from samples collected in Cambodia and Madagascar (Table 1). The sample (throat swab) from Cambodia was collected from a Lyle's flying fox (*Pteropus lylei*; family Pteropodidae) captured and released in a tree roost located in the village/municipality of Svay Sach Phnum (Srey Santhor District, Kampong Cham Province; 11° 54.946' N 105° 10.907' E). Samples from Madagascar (throat swabs) were collected from endemic

Madagasy fruit bats (*Eidolon dupreanum*; family Pteropodidae) captured and released in the Angavokely Caves (Nandihizana Carion Municipality, Manjakandriana District; 18° 55.947' S 47° 45.445' E) and Angavobe Caves (Sabotsy Anjiro Municipality, Moramanga District; 18° 55.083' S 47° 56.616' E). Isolates from these two countries were obtained during surveys for henipaviruses (Reynes *et al.*, 2005; lehlé *et al.*, 2007). They were suspected to belong to the family *Herpesviridae* because they induced cytopathic effects (CPE) on Vero E6 cells compatible with those produced by alphaherpesviruses (rapid mass destruction of the cells associated with polykaryocytosis and/or rounded cells). Furthermore, margination of the chromatin in the nucleus and vacuolization of the cytoplasm were observed in Giemsa-stained infected Vero E6 cells (results not presented).

Isolates were propagated in Vero E6 cells to obtain virus-containing supernatants for molecular studies. DNA was extracted from these supernatants using the classical phenol/chloroform method or with the QIAamp DNA blood mini kit (Qiagen) following the manufacturer's instructions. DNA was then submitted to PCR using the degenerate consensus primers DFASA and GDTD1B targeting the UL30 catalytic subunit region of the DNA polymerase gene (Rose *et al.*, 1997) using PCR cycling conditions described previously (Lacoste *et al.*, 2001). PCR products of the expected size (~518 bp) were obtained from DNA of all isolates. The amplification products were sequenced on both strands by Cogenics (Meylan, France). Unverified sequences and chromatograms returned to us by the company were compared and corrected when needed. Sequences were aligned and fragments of 465 bp shared by all 13 samples and encoding amino acids 724 to 878 of the catalytic subunit of DNA polymerase (positions according to the DNA polymerase sequence from *Human herpesvirus 1*; GenBank accession no. CAA32323) were selected for the rest of the analysis. The partial nucleotide sequences from the Cameroonian isolates were identical, as were those from all nine Malagasy isolates. These results are not surprising, since the isolates were obtained in each country at the same locality, within a short period and from the same host. Interestingly, there was only one nucleotide difference (among 465) between the Cameroonian and Malagasy nucleotide sequences and their amino acid sequences were identical. Sequences from the Brazilian, Cambodian and Central African Republic isolates were more distant, suggesting that these three isolates are representative of three other viral species (Table 2). Database searches using the BLAST web server demonstrated that these sequences were novel and most similar to those of DNA polymerases of the subfamily *Alphaherpesvirinae*.

A phylogenetic tree was constructed with the amino acid sequences of one isolate from each country (Brazil, Madagascar, Cameroon and Central African Republic) and the available sequences of known herpesviruses from other host species, particularly those belonging to the subfamily *Alphaherpesvirinae*. Phylogenetic analysis of all the sequences was conducted with MEGA version 4 (Tamura *et al.*, 2007), using the neighbour-joining

method with the Poisson model correction. This analysis confirms the affiliation of these isolates with the subfamily *Alphaherpesvirinae* and placed all the isolates in the genus *Simplexvirus* (Fig. 1).

Bats have been described recently as natural hosts for betaherpesviruses and gammaherpesviruses in Europe (Wibbelt *et al.*, 2007). The data presented in this paper expand the taxonomic range of herpesviruses within bat hosts to the subfamily *Alphaherpesvirinae*. Although not all of the virus genome was sequenced, four new members in the genus *Simplexvirus* have been uncovered. The nature of the CPE induced on Vero E6 cells by the Cambodian and Malagasy isolates supports their placement in this genus (Roizman, 1996). Furthermore, these data confirm and extend the geographical distribution of herpesvirus in bats to three more continents (Africa, South America and Asia) and indicate the presence of these viruses in frugivorous bats of the families Pteropodidae and Phyllostomidae.

Interestingly, the partial sequences of the Malagasy and Cameroonian isolates were identical, suggesting that they belong to the same member of the genus *Simplexvirus*. The Malagasy and Cameroonian isolates were obtained from the bats *Eidolon dupreanum* and *Eidolon helvum*, respectively, the only two recognized members of this genus. During the henipavirus survey in Madagascar, we isolated the orbivirus Ife and detected neutralizing antibodies against the Lagos bat lyssavirus in *E. dupreanum* (J.-M. Reynes, unpublished data). These two viruses have also been isolated from bats belonging to *E. helvum*, which is strictly African in distribution (Calisher *et al.*, 2006). The sharing of these viruses by the only two species in this genus is extraordinary, as the two populations are isolated from one another by the Mozambique Channel, a distance of 450 km between the closest points of Africa and Madagascar, and no exchange has been reported (S. M. Goodman, personal communication).

None of the herpesviruses described in European bats could be related consistently to a pulmonary lesion or any other distinct histopathology in the sampled animals (Wibbelt *et al.*, 2007). The Cambodian isolate was obtained from an apparently healthy bat. There is no information on the health status of the sampled Cameroonian, Central African Republic and Brazilian bats. The Malagasy isolates were obtained from apparently healthy bats. However, in Madagascar, we noticed that the herpesviruses isolates were obtained from throat swabs collected in 2006 when keratitis (sometimes ulcerative) was observed in 15 of the 143 bats sampled (virus isolation tested negative using throat swabs from these 15 animals). No herpesvirus was isolated from throat swabs collected from 109 animals in 2007, when keratitis was observed in only one animal. This association is striking, since alphaherpesviruses such as *Human herpesvirus 1* and *Feline herpesvirus 1* have been responsible for keratitis in their host (Andrew, 2001).

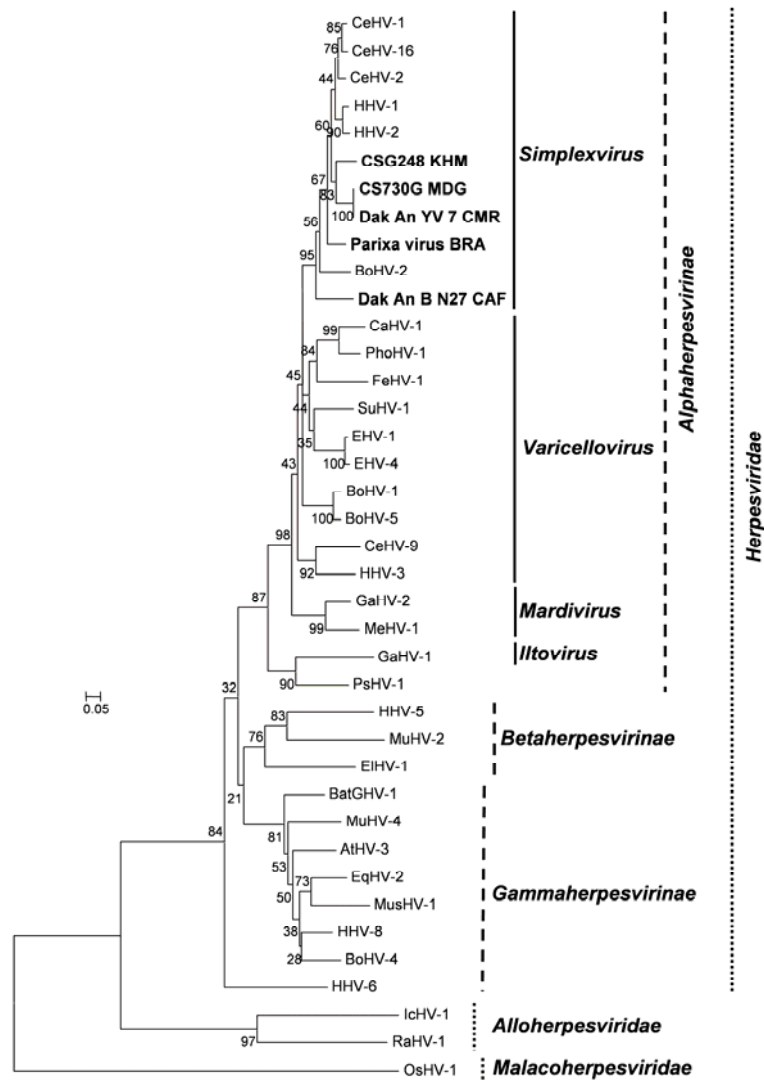
Sampling in Madagascar continues, and virus isolation will be attempted from corneal swabs collected from bats exhibiting keratitis.

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**Fig. 1.** Phylogenetic tree based on deduced amino acid sequences of the UL30 fragment. Novel bat herpesviruses are highlighted in bold. BRA, Brazil; CAF, Central African Republic; CMR, Cameroon; KHM, Cambodia; MDG, Madagascar. Bootstrap percentages (from 1000 resamplings) are indicated at each node. Bar, evolutionary distance of 0.05. Virus name abbreviations and accession numbers of reference sequences are: AtHV-3 (*Ateline herpesvirus 3*; GenBank accession no. AAC95533), BatGHV-1 (*Bat gammaherpesvirus 1*; DQ788623), BoHV-1 (*Bovine herpesvirus 1*; AJ004801), BoHV-2 (AF181249), BoHV-4 (AF318573), BoHV-5 (AY261359), CaHV-1 (*Canine herpesvirus*; AY949827), CeHV-1 (*Cercopithecine herpesvirus 1*; AF533768), CeHV-2 (NC\_006560), CeHV-9 (AF275348), CeHV-16 (AY168637), EHV-1 (*Equid herpesvirus 1*; AY665713), EHV-2 (U20824), EHV-4 (AF030027), EIHV-1 (*Elephantid herpesvirus 1*; AF322977), FeHV-1 (*Feline herpesvirus 1*; AJ224971), GaHV-1 (*Gallid herpesvirus 1*; NC\_006623), GaHV-2 (AF243438), HHV-1 (*Human herpesvirus 1*; X14112), HHV-2 (Z86099), HHV-3 (X04370), HHV-5 (X17403), HHV-6 (X83413), HHV-8 (U93872), IchHV-1 (*Ictalurid herpesvirus 1*; M75136), MeHV-1 (*Meleagrid herpesvirus 1*; AF291866), MuHV-2 (*Murid herpesvirus 2*; AY728086), MuHV-4 (U97553), MusHV-1 (*Mustelid herpesvirus 1*; AF376034), OsHV-1 (*Ostreid herpesvirus 1*; AY509253), PhoHV-1 (*Phocid herpesvirus 1*; U92269), PsHV-1 (*Psittacid herpesvirus 1*; AY372243), RaHV-1 (*Ranid herpesvirus 1*; DQ665917) and SuHV-1 (*Suid herpesvirus 1*; BK001744).

**Table 1.** Bat virus isolates analysed

The genera *Eidolon* and *Pteropus* belong to the family Pteropodidae, whereas *Lonchophylla* belongs to the family Phyllostomidae.

Isolate	Isolation			Sampling	
	Date	Host species	Country	Date	Sample
CS730G	3 July 2006	<i>Eidolon dupreanum</i>	Madagascar	10 May 2006	Throat swab
CS732G	11 July 2006	<i>E. dupreanum</i>	Madagascar	10 May 2006	Throat swab
CS735G	11 July 2006	<i>E. dupreanum</i>	Madagascar	10 May 2006	Throat swab
CS736G	13 July 2006	<i>E. dupreanum</i>	Madagascar	10 May 2006	Throat swab
CS739G	13 July 2006	<i>E. dupreanum</i>	Madagascar	10 May 2006	Throat swab
CS745G	14 July 2006	<i>E. dupreanum</i>	Madagascar	10 May 2006	Throat swab
CS754G	14 July 2006	<i>E. dupreanum</i>	Madagascar	21 June 2006	Throat swab
CS840G	27 September 2006	<i>E. dupreanum</i>	Madagascar	13 September 2006	Throat swab
CS847G	28 September 2006	<i>E. dupreanum</i>	Madagascar	13 September 2006	Throat swab
Dak An Y 6	21 April 1971	<i>Eidolon helvum</i>	Cameroon	13 April 1971	Organs
Dak An Y 7	21 April 1971	<i>E. helvum</i>	Cameroon	13 April 1971	Organs
Dak An Y 9	20 April 1971	<i>E. helvum</i>	Cameroon	13 April 1971	Organs
Dak An B N27	August 1965	Unidentified	Central African Republic	20 July 1965	Salivary glands
CSG248	8 March 2004	<i>Pteropus lylei</i>	Cambodia	10 October 2003	Throat swab
Parixa virus (Be An 422840)	Not known	<i>Lonchophylla thomasi</i>	Brazil	3 May 1984	Blood

**Table 2.** Differences of partial DNA polymerase gene and deduced protein sequences of bat herpesviruses

Values above the diagonal are percentages of nucleotide sequence divergence and values below the diagonal are percentages of amino acid sequence divergence.

Isolate	Country	1	2	3	4	5
1. CS732G	Madagascar	(0)	0	27.7	13.6	21.3
2. Dak An Y 7	Cameroon	0.2	(0)	27.7	13.6	21.3
3. Dak An B N27	Central African Republic	31.2	31.2	(0)	25.8	22.6
4. CSG248	Cambodia	24.1	23.9	30.8	(0)	20.6
5. Parixa virus	Brazil	25.6	25.6	27.6	24.7	(0)