Seven novel gammaherpesviruses (GHV) and one novel betaherpesvirus were discovered in seven different European bat species (order Chiroptera, family Vespertilionidae) with a pan-herpesvirus PCR assay, targeting the DNA polymerase (DPOL) gene. The sequences of six bat GHV were similarly related to members of the gammaherpesvirus genera *Percavirus* and *Rhadinovirus*. The seventh GHV was related to the porcine lymphotropic herpesvirus 1 (genus *Macavirus*). The betaherpesvirus appeared to be a distant relative of human cytomegalovirus. For three bat GHV a 3.6 kbp locus was amplified and sequenced, spanning part of the glycoprotein B gene and the majority of the DPOL gene. In phylogenetic analysis, the three bat GHV formed a separate clade with similar distance to the *Percavirus* and *Rhadinovirus* clades. These novel viruses are the first herpesviruses to be described in bats.

Since the recent advent of henipavirus infections and SARS, interest in the role of bats as hosts for pathogens has markedly increased. With the exception of research on bat lyssaviruses, having been studied worldwide (Johnson et al., 2006), most virological investigations in bats have been limited to a particular infectious agent implicated in a geographically localized disease outbreak (Bowden et al., 2001; Chua et al., 2002; Lau et al., 2005). Additionally, a large number of less important viruses have been found in bats in the Americas, Africa, Asia and Australia (Calisher et al., 2006; Wibbelt et al., 2007), but bats from Europe have not been studied. Furthermore, the existence of bat herpesviruses (bat HV) has so far not been reported, except in an earlier study of Tandler (1996), who described cytomegalovirus-like particles in salivary glands of the little brown bat (*Myotis lucifugus*) by light and electron microscopy. Molecular studies using PCR-based methods combined with sequence analysis are lacking.

In the present study, we performed a molecular search for bat HV, as a part of a broader study concerning the occurrence of histopathological changes and associated infectious pathogens in European bats (Wibbelt and others, unpublished). Free-ranging, moribund or dead European bats were collected by bat conservationists. Depending on their state of fixation or decomposition, 25 animals from eight bat species (*Eptesicus serotinus*, *Myotis myotis*, *Myotis nattereri*, *Nyctalus leisleri*, *Nyctalus noctula*, *Pipistrellus nathusi*, *Pipistrellus pipistrellus* and *Plecotus auritus*; order Chiroptera, family Vespertilionidae) were considered suitable for further investigations. Twenty-one animals originated each from a different location within the greater Berlin area (north-east Germany), while four animals were collected at different places near Freiburg (south-west Germany). Carcasses were necropsied, and tissue specimens of all major organs were subjected to histopathological examination. Pneumonia of varying degree was the major finding in 50% of all carcasses. Specimens of affected and unaffected lungs were selected to be tested by PCR for the presence of herpesviruses. For this purpose, DNA was isolated using the QIAamp DNA Blood Mini Kit (Qiagen), according to the manufacturer’s instructions.

Before assaying for herpesviruses, identification of the bat species was confirmed or corrected by amplification and sequencing of the cytochrome B (*cytB*) gene. Species identification by means of the *cytB* gene is a commonly used technique (Linacre & Lee, 2005), and roughly 1000 *cytB* sequences of bats of the Vespertilionidae are available in GenBank for comparison. Here, we amplified 241 bp of the *cytB* gene with degenerate primers (primers are listed in Supplementary Table S1, available with the online version of this paper), and the amplimers were sequenced (data not shown).

For initial detection of herpesviruses, a partial DNA polymerase (DPOL) gene sequence of a few hundred base pairs is generally amplified with degenerate primers or deoxynosine-substituted primers (VanDevanter et al., 1996; Ehlers et al., 1999). In the present study, the pan-herpes consensus-PCR was carried out with primers which were both degenerate and deoxynosine-substituted (deg/di) i.e. inosine was introduced at wobble positions like a fifth base (Chmielewicz et al., 2003). Amplimers of 166–178 bp (excluding primer binding sites) of the DPOL gene were obtained, the length depending on the herpesvirus
amplified. Samples with little or no amplification product were reanalysed 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 AmpliTaq Gold (Applied Biosystems) was increased 2-fold. PCR product purification, direct sequencing with dye terminator chemistry as well as nucleotide and amino acid sequence analysis were carried out as described previously (Goltz et al., 2002).

Eight different DPOL gene sequences, originating from seven gammaherpesviruses and one betaherpesvirus, were detected in 7 out of 8 European bat species and deposited in GenBank (Table 1 and Fig. 1a). For the purpose of this report, the novel viruses were named as bat beta- or gammaherpesviruses (BatBHV or BatGHV), followed by a number which indicates the chronological order of detection of gammaherpesviruses (BatBHV or BatGHV), followed by a number which indicates the chronological order of detection (Table 1), example: Bat gammaherpesvirus 1 (BatGHV-1).

The seven BatGHV sequences were compared pairwise on the amino acid sequence level with the CLUSTAL W module (Table 1), example: Bat gammaherpesvirus 1 (BatGHV-1).

Phylogenetic trees were then constructed with neighbour-joining and maximum-likelihood analysis, as performed earlier (Ehlers & Lowden, 2004). Six bat HV (BatGHV-1, BatGHV-2, BatGHV-4 to BatGHV-7) formed a separate clade within the Gammaherpesvirinae (Fig. 1b). The virus BatGHV-3 branched separately, together with the porcine lymphotropic herpesvirus 1 (PLHV-1), which is a member of the proposed genus Macavirus (McGeoch et al., 2006) within the Gammaherpesvirinae (Fig. 1b). The virus BatBHV-1 revealed the highest identity value (45 %) to the betaherpesvirus tupata herpesvirus 1 (Fig. 1a; Table 1) and branched as a betaherpesvirus (Fig. 1b).

All gammaherpesvirus-positive samples were also tested with the deg/dl nested-primer set RH-gB (Supplementary Table S1), which targets the gB genes of members of the Gammaherpesvirinae subfamily. Second-round amplification products had a calculated length of approximately 450 bp (without primer-binding sites). For three gammaherpesviruses (BatGHV-1, BatGHV-4 and BatGHV-5), gB amplimers of the expected size were obtained and sequenced. Comparison with the gB genes of other herpesviruses revealed high similarity to the Gammaherpesvirinae (not shown). An attempt to amplify a partial gB sequence of BatBHV-1 with deg/dl nested primers, targeting gB sequences of betaherpesviruses, failed. This was probably due to the generally higher sequence diversity within the Betaherpesvirinae, making the design of degenerate primers difficult.

We then intended to prove that the partial DPOL and gB sequences, obtained from the same BatGHV-positive sample, were indeed derived from the same virus genome. For this purpose, we connected the partial DPOL and gB sequences with long-distance PCR, using the TaKaRa-Ex PCR system (Takara Bio) according to the manufacturer’s instructions. Amplimers were generated by nested PCR with specific sense primers derived from the partial gB sequences and specific anti-sense primers derived from the partial DPOL sequences (primers are listed in Supplementary Table S2, available with the online version of this paper). For the second round, 1 μl of the first round mix was used as a template. For BatGHV-1, BatGHV-4 and BatGHV-5, amplimers of expected size were obtained. The sequences were found to contain the 3’ part of the gB gene and the 5’ part of the DPOL gene. Three final contiguous sequences of about 3.6 kbp were generated and deposited in GenBank (accession numbers in Table 1).

In BLASTX searches (Altschul et al., 1990) with gB nucleic acid sequences and subsequent pairwise amino acid sequence comparisons (approx. 370 aa) with MacVector, BatGHV-1, 4 and 5 revealed the highest percentage of identity to the badger herpesvirus (Table 1), a member of the proposed genus Percavirus (McGeoch et al., 2006). However, with DPOL sequences (approx. 760 aa), slightly higher percentages were seen with members of the genus Rhadinovirus (herpesvirus saimiri, bovine herpesvirus 4, rhadinovirus of black rhinoceros) (Table 1).

Phylogenetic analysis with concatenated gB and DPOL sequences (approx. 1130 aa) confirmed that bat gammaherpesviruses form a new group within the Gammaherpesvirinae. However, their exact phylogenetic position within this subfamily remained unresolved. The clade revealed a similar distance to equine herpesvirus 2 (EHV-2) and the gammaherpesvirus from badger (both genus Percavirus) on the one hand and to gammaherpesviruses of the genus Rhadinovirus on the other (Fig. 1b). Further characterization of the bat HV is needed for their taxonomic assignment.

Primer sets specific for each novel herpesvirus were used to re-evaluate each bat DNA sample by PCR (Supplementary Table S1, available with the online version of this paper). All eight pre-isolated bat HV sequences could be reamplified and were 100 % identical to the sequences previously amplified with the deg/dl primers as confirmed by sequencing. Five previously negative bats were positive for one or more bat HV when tested with the virus-specific primers. Three viruses were found only in a single bat species, while five viruses were found in more than one bat species. In total, 15 out of 25 bats were found to contain varying numbers of eight different HV from two herpesvirus families (Table 1). Fourteen of these animals originated from north-east Germany, while one animal came from south-west Germany. As all animals were found at different locations and on different dates, it is unlikely that individuals of the same species originated from the same roost population. Therefore, the high number of positive individuals indicated a wide distribution of...
Table 1. Novel bat herpesviruses

<table>
<thead>
<tr>
<th>Virus name (abbreviation)</th>
<th>Host species</th>
<th>Positive animals* number</th>
<th>Accession number</th>
<th>Most similar bat HV/most similar HV of other mammals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bat gammaherpesvirus 1 (BatGHV-1)</td>
<td>Serotine bat† (&lt;i&gt;Eptesicus serotinus&lt;/i&gt;)</td>
<td>1/4</td>
<td>DQ788623</td>
<td>76 % (BatGHV-7)/67 % (Herpesvirus saimiri) gB: 60 % (BatGHV-4)/51 % (Badger RHV‡) DPOL: 65 % (BatGHV4)/59 % (Black rhinoceros RHV)</td>
</tr>
<tr>
<td></td>
<td>Natterer’s bat§ (&lt;i&gt;Myotis nattereri&lt;/i&gt;)</td>
<td>1/4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nathusius’ pipistrelle bat§ (&lt;i&gt;Pipistrellus nathusii&lt;/i&gt;)</td>
<td>1/1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Common pipistrelle bat§ (&lt;i&gt;Pipistrellus pipistrellus&lt;/i&gt;)</td>
<td>2/9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bat gammaherpesvirus 2 (BatGHV-2)</td>
<td>Natterer’s bat† (&lt;i&gt;Myotis nattereri&lt;/i&gt;)</td>
<td>2/4</td>
<td>DQ788625</td>
<td>64 % (BatGHV-5 and 7)/61 % (Herpesvirus saimiri)</td>
</tr>
<tr>
<td></td>
<td>Greater mouse eared bat§ (&lt;i&gt;Myotis myotis&lt;/i&gt;)</td>
<td>1/1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bat gammaherpesvirus 3 (BatGHV-3)</td>
<td>Noctule bat† (&lt;i&gt;Nyctalus noctula&lt;/i&gt;)</td>
<td>1/2</td>
<td>DQ788626</td>
<td>49 % (BatGHV-5)/51 % (Porcine lymphotropic herpesviruses 1 and 2)</td>
</tr>
<tr>
<td></td>
<td>Natterer’s bat§ (&lt;i&gt;Myotis nattereri&lt;/i&gt;)</td>
<td>1/4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Greater mouse eared bat§ (&lt;i&gt;Myotis myotis&lt;/i&gt;)</td>
<td>1/1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bat gammaherpesvirus 4 (BatGHV-4)</td>
<td>Noctule bat† (&lt;i&gt;Nyctalus noctula&lt;/i&gt;)</td>
<td>1/2</td>
<td>DQ788627</td>
<td>85 % (BatGHV-6)/67 % (Chimpanzee RHV2) gB: 78 % (BatGHV-5)/51 % (Badger RHV) DPOL: 81 % (BatGHV-5)/58 % (Bovine herpesvirus 4)</td>
</tr>
<tr>
<td></td>
<td>Natterer’s bat§ (&lt;i&gt;Myotis nattereri&lt;/i&gt;)</td>
<td>1/4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bat gammaherpesvirus 5 (BatGHV-5)</td>
<td>Nathusius’ pipistrelle bat† (&lt;i&gt;Pipistrellus nathusii&lt;/i&gt;)</td>
<td>1/1</td>
<td>DQ788629</td>
<td>76 % (BatGHV-4)/69 % (Herpesvirus saimiri) gB: 78 % (BatGHV-4)/52 % (Badger RHV) DPOL: 81 % (BatGHV-4)/60 % (Herpesvirus saimiri)</td>
</tr>
<tr>
<td>Bat gammaherpesvirus 6 (BatGHV-6)</td>
<td>Common pipistrelle bat† (&lt;i&gt;Pipistrellus pipistrellus&lt;/i&gt;)</td>
<td>2/9</td>
<td>DQ788630</td>
<td>85 % (BatGHV-4)/67 % (Hylobates leucogenys rhadinovirus 2)</td>
</tr>
<tr>
<td>Bat gammaherpesvirus 7 (BatGHV-7)</td>
<td>Common long eared bat† (&lt;i&gt;Plecotus auritus&lt;/i&gt;)</td>
<td>1/2</td>
<td>DQ788628</td>
<td>76 % (BatGHV-1)/67 % (Herpesvirus saimiri)</td>
</tr>
<tr>
<td>Bat betaherpesvirus 1 (BatBHV-1)</td>
<td>Natterer’s bat† (&lt;i&gt;Myotis nattereri&lt;/i&gt;)</td>
<td>2/4</td>
<td>DQ788624</td>
<td>25 % (BatGHV-2)/45 % (Tupaia herpesvirus 1)</td>
</tr>
<tr>
<td></td>
<td>Common pipistrelle bat§ (&lt;i&gt;Pipistrellus pipistrellus&lt;/i&gt;)</td>
<td>2/9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Number of positive animals vs number of tested animals.
†Host species virus-positive after primary PCR screening with degenerate primers and secondary PCR screening with specific primers.
‡RHV, rhadinovirus.
§Host species virus-positive after secondary PCR screening with specific primers.

Different bat herpesviruses. Furthermore, the infection of different vespertilionid hosts with apparently the same herpesvirus could be explained by the fact that the novel herpesviruses identified have more than one primary host among the Vespertilionidae, possibly fostered by close inter-species contacts in roosts.

Herpesviruses have been discovered in mammals, birds, reptiles, amphibians, fishes and molluscs, and to date, the number of known distinct herpesviruses exceeds 200 (Ehlers, 2008). Despite this knowledge, the majority of potential animal hosts for herpesviruses has so far not been investigated. This is particularly true for mammalian
Fig. 1. Multiple alignment and phylogenetic analysis of the novel bat herpesviruses. (a) Nucleic acid sequences of beta- and gammaherpesviruses were translated and aligned with the CLUSTAL W module of MacVector (version 8.0). Identical and similar amino acids are represented by bold and standard letters, respectively, and boxed. Gaps are represented by dashes. Sequences of known herpesviruses were taken from GenBank (accession numbers in legend of Fig. 1b). (b) A phylogenetic tree was constructed using a multiple alignment of approximately 1000 aa, consisting of concatenated gB and DPOL sequences of the novel bat gammaherpesviruses BatGHV-1, BatGHV-4 and BatGHV-5 and the following additional gammaherpesviruses, available in GenBank: AlHV-1 (alcelaphine herpesvirus 1, accession no. AF005370); badger herpesvirus (AF376034); BoHV-4 (bovine herpesvirus 4, NC_002665); EBV (Epstein–Barr virus, X00784); EHV-2 (equine herpesvirus 2, U20824); HCMV (human cytomegalovirus, X17403); HHV-8 (human herpesvirus 8, U93872); HVS (herpesvirus saimiri, X64346); MHV-68 (murine herpesvirus 68, U97553); PLHV-1 (AF478169) and RRV (rhesus monkey rhadinovirus, AF083501). The branching of BatGHV-2, BatGHV-3, BatGHV-6 and BatGHV-7 as well as BatBHV-1 was taken from a shorter DPOL alignment and is indicated by dashed lines. The alignment was analysed with the neighbour-joining method using the program MacVector. A rooted phylogram is shown, with HCMV as the outlier. The branch length is proportional to evolutionary distance (scale bar). Bootstrap values (100-fold) are indicated at the nodes of the tree to the left of the vertical divider. In addition, the alignment was analysed with the program TREE - PUZZLE (version 5.0). Support values, estimated by the quartet puzzling (QP) tree search and expressing the QP reliability as a percentage, are indicated to the right of the vertical divider. The novel viruses are highlighted in bold. Herpesvirus genera and families are indicated.
families with high numbers of different species like Rodentia or Chiroptera, which are the two largest vertebrate families with the highest numbers of species. The over 1100 species of bats make up around 20% of all living mammalian species (Simmons, 2005). In this study, seven gamma- and one betaherpesvirus were discovered in 15 individual bats. None of these viruses could be related consistently to a pulmonary lesion or any other distinct histopathological finding. Since herpesviruses enter a latent state after primary infection and reactivate only under certain conditions, we may have exclusively detected latent bat HV. However, the aetiological association of bat herpesviruses with pulmonary disease can presently not be excluded and remains to be elucidated.

In summary, this study is the first comprehensive study on herpesviruses in bats. It describes eight new herpesviruses and allows the first detailed insight into their genetic relationships.

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References


