Detection of novel gammaherpesviruses from fruit bats in Indonesia

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Abstract

Bats are an important natural reservoir of zoonotic viral pathogens. We previously isolated an alphaherpesvirus in fruit bats in Indonesia, and here establish the presence of viruses belonging to other taxa of the family *Herpesviridae.* We screened the same fruit bat population with pan-herpesvirus PCR and discovered 68 sequences of novel gammaherpesvirus, designated 'megabat gammaherpesvirus' (MgGHV). A phylogenetic analysis of approximately 3.4 kbp of continuous MgGHV sequences encompassing the glycoprotein B gene and DNA polymerase gene revealed that the MgGHV sequences are distinct from those of other reported gammaherpesviruses. Further analysis suggested the existence of coinfections of herpesviruses in Indonesian fruit bats. Our findings extend our understanding of the infectious cycles of herpesviruses in bats in Indonesia and the phylogenetic diversity of the gammaherpesviruses.

Text

Outbreaks of emerging and re-emerging zoonoses have compromised public health and damaged the global economy in recent years. Bats are known to be carriers of lyssaviruses, henipaviruses, severe acute respiratory syndrome-like coronaviruses, and filoviruses, so they are regarded as an important natural reservoirs of zoonotic viral pathogens [1-5].

Herpesviruses are enveloped, double-stranded DNA viruses belonging to the family *Herpesviridae*. These viruses include three genera: *Alphaherpesvirinae*, *Betaherpesvirinae,* and *Gammaherpesvirinae*. *Gammaherpesvirinae* contains viruses that are oncogenic to humans, including Human gammaherpesvirus 4 (also known as Epstein-Barr virus) and Human gammaherpesvirus 8 (also known as Kaposi's sarcoma-associated herpesvirus). Therefore, the viruses of the genus *Gammaherpesvirinae* are important in human health.

Many gammaherpesvirus sequences have recently been detected in a broad range of bat species worldwide [6-9]. Myotis gammaherpesvirus 8 was isolated from a tumor cell line derived from *Myotis velifer incautus* (mouse-eared bat), and this virus has been shown to cause cytopathic effects in Vero cells and can replicate in some human cell lines [10, 11].

In a previous study, we reported a high prevalence of fruit bat alphaherpesvirus 1 (FBAHV1) in fruit bats in Indonesia [12]. Here, to extend our understanding of the herpesviruses carried by fruit bats, we used PCR to screen for a broad spectrum of herpesviruses, and followed by phylogenetically analyzed the sequences detected.

In this study, 183 bat tissue samples were analyzed: 69 from *Pteropus vampyrus* (large flying fox), 61 from unassigned *Pteropus* bats (*Pteropus* sp.), 17 from *Dobsonia moluccensis* (Moluccan bare-backed fruit bat), and 36 from *Acerodon celebensis* (Sulawesi fruit bat). The samples were collected in the Lima Puluh Kota, Magelang, Panjalu, Paguyaman, Popayato, Sidrap, Soppeng, and Surabaya regions of Indonesia in 2010-2014, as shown in Fig. 1 and Table 1. Several healthy bats were captured from flocks at each location and euthanized with an overdose of ketamine and xylazine, with the permission of the Directorate General of Livestock and Animal Health Services, Ministry of Agriculture, Republic of Indonesia. All procedures were approved by the Animal Care and Use Committee of the Veterinary Teaching Hospital, Bogor Agricultural University (permit number 05-2010 RSHP-IPB). The samples were stored at -80°C and have been used previously to screen for FBAHV1 and other viruses [12-15]. The bat species were identified according to their morphological characteristics, and a nucleotide sequence analysis of their mitochondrial 16S rRNA and cytochrome *b* genes, as previously described [12, 15]. The mitochondrial nucleotide sequences of *Pteropus* sp. showed greatest identity (96% 16S rRNA and 95% cytochrome *b* genes) to the corresponding sequences of *P. hypomelanus* (small flying fox; accession numbers AF069537 and AB062472). Therefore, we concluded that these bats were closely related to *P. hypomelanus*, but should be classified as a different species.

Total DNA was extracted from spleen tissue samples with DNAzol (Molecular Research Center) or the QIAamp DNA Mini Kit (Qiagen). We screened for

herpesviruses with semi-nested PCR using TaKaRa Ex Taq Hot Start Version (Takara Bio) and degenerate primers targeting the DNA polymerase (*DPOL*) gene of the herpesviruses [16]. This primer set has been used to detect herpesviruses in a broad range of wild animals [17-19]. The PCR products, of approximately 250 bp, were purified from the gel after electrophoresis and sequenced directly with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequences were analyzed with BLASTN, and 71 of the 183 tested samples were positive for herpesvirus sequences. The BLASTN results suggested that the 67 of these 71 sequences (172 bp in length) originated from gammaherpesviruses and that the remaining four sequences (181 bp in length) originated from betaherpesviruses, as summarized in Table S1 (available in the online Supplementary Material). Therefore, the origins of these herpesvirus sequences were designated 'megabat gammaherpesvirus' (MgGHV) and 'megabat betaherpesvirus' (MgBHV), respectively. The *DPOL* sequences determined were deposited in GenBank under accession numbers LC268906-LC268972. The sequences of MgGHV were detected in the bat samples collected at every location that we sampled, suggesting that MgGHVs are geographically distributed throughout Indonesia, as summarized in Figure 1 and Table 1.

Because the majority of the sequences belonged to MgGHV, distributed geographically throughout Indonesia, we focused our investigation on MgGHV. A multiple-sequence alignment by CLUSTAL W and a subsequent Maximum likelihood phylogenetic analysis of the partial MgGHV *DPOL* sequences were performed by the MEGA 7 program [20]. The MgGHVs were phylogenetically

divided into seven groups (MgGHV Group-1 to -7), as shown in Fig. 2(a). The MgGHV sequences clustered according to the bat species in which they were detected, except MgGHV Group-1, which contained sequences detected in *Pteropus* sp. and *Acerodon celebensis*. All of the MgGHV groups were phylogenetically related to previously described gammaherpesviruses detected in *Pteropus* species. MgGHV Group-1, -2 and -3 formed a cluster with Pteropus giganteus gammaherpesvirus isolate-3 (PgHV-3); MgGHV Group-4 and -5 formed a cluster with PgHV-2; and MgGHV Group-6 was closely related to PgHV-1. These PgHVs were detected in *P. giganteus* in Bangladesh [21]. MgGHV Group-7 contained a single sequence that was identical to that of Pteropine herpesvirus 2, which was detected in *Pteropus vampyrus* kept in a captive breeding facility in the United States [22].

To investigate the MgGHV phylogeny in more detail, we additionally screened for glycoprotein B (*gB*) gene, which is located upstream from the *DPOL* gene, and subsequent long-distance PCR (LD-PCR). Both of *DPOL* and *gB* are commonly used for the phylogenetic analysis of the gammaherpesviruses [23, 24]. The target sequence of the LD-PCR was approximately 3.4 kbp in length and flanked by the partial *gB* and *DPOL* sequences obtained from the screening [17, 25].

First, we used nested PCR to screen for the gammaherpesvirus *gB* gene using Platinum Taq DNA Polymerase (Invitrogen) and degenerate primers, as previously described [26]. With this procedure, the 417-456 bp partial *gB* sequences were obtained from 16 of the 67 spleen DNA samples for gammaherpesvirus *DPOL,* and a BLASTN analysis showed that all of them were

151 derived from gammaherpesviruses, as summarized in Table 1. The *gB* sequences
152 determined were deposited in the GenBank under accession numbers
153 LC268973-LC268980 and LC268982-LC268986. A phylogenetic analysis of these
154 *gB* sequences revealed that the MgGHVs formed a discrete cluster, separate from
155 known gammaherpesviruses clades, as shown in Fig. 2(b). Based on the *gB*
156 phylogeny, the MgGHVs were tentatively assigned to six groups, MgGHV
157 Group-A to -F. MgGHV Group-B, -C, -E and -F were congruent with the bat
158 species in which they were detected, whereas MgGHV Group-A and -D contained
159 sequences detected in both *Pteropus vampyrus* and *Pteropus* sp., as summarized
160 in Table S1. Remarkably, the phylogenetic relationships of each MgGHV group
161 were not consistent between the phylogenetic trees constructed with the *DPOL*
162 and *gB* sequences, as shown in Fig. 2 (a, b). For instance, *DPOL* from several bat
163 samples was assigned to MgGHV Group-1, whereas the *gB* sequences detected
164 in the same samples were assigned to several different groups (MgGHV Group-A,
165 B, C and F), as summarized in Table S2 (available in the online Supplementary
166 Material). These observations suggest the presence of coinfections with different
167 MgGHVs in individual fruit bats. Mühldorfer *et al.* have previously reported
168 coinfections of BatGHV-3, -4, and -5 in *Nyctalus noctula* (noctule), but this is the
169 first report of coinfections of gammaherpesviruses in *Pteropus vampyrus*,
170 *Pteropus* sp. and *Dobsonia moluccensis* [7]. A genomic recombination event has
171 previously been reported in the human gammaherpesviruses [27]. Therefore, it is
172 possible that different gammaherpesviruses might interact each other and play an
173 important role in their survival or evolution in bats. Therefore, coinfection warrants

174 attention in studies of the prevalence or evolutionary history of
175 gammaherpesviruses in bats in future epidemiological researches.
176 LD-PCR was then performed on the samples doubly positive for samples
177 *DPOL* and *gB*. Of the 16 samples tested, four were positive for the 3,351-3,544 bp
178 LD-PCR target sequence between *DPOL* and *gB*. The origins of these *DPOL-gB*
179 sequences were designated MgGHV_IFB11-41 (LC268987), MgGHV_IFB12-05
180 (LC268988), MgGHV_IFB12-16 (LC268989), and MgGHV_IFB13-11 (LC268990).
181 A phylogenetic analysis based on the *DPOL-gB* sequences grouped the viruses
182 into two clusters, as shown in Fig. 2 (c). MgGHV_IFB11-41 and MgGHV_IFB13-11
183 were detected in *Pteropus* sp., and were phylogenetically located relatively close
184 to Murid gammaherpesvirus 4. In an analysis of nucleotide identity with the
185 GENETYX software v.10 (Genetyx Corporation), MgGHV_IFB11-41 and
186 MgGHV_IFB13-11 showed highest identity (61%-62%) to Diceros bicornis
187 gammaherpesvirus 1. MgGHV_IFB12-05 and MgGHV_IFB12-16 were detected in
188 *Dobsonia moluccensis*, and formed a cluster with Hexaprotodon liberiensis
189 gammaherpesvirus 1 and Human gammaherpesvirus 4. MgGHV_IFB12-05 and
190 MgGHV_IFB12-16 shared highest identity (66%) with Hexaprotodon liberiensis
191 gammaherpesvirus 1. All four MgGHVs identified here were segregated from
192 currently known gammaherpesviruses. To date, the nucleotide sequences
193 corresponding to the *DPOL-gB* region are available for six bat
194 gammaherpesviruses: Eptesicus serotinus rhadinovirus 1, Nyctalus noctula
195 rhadinovirus 2, Myotis gammaherpesvirus 8, Myotis ricketti herpesvirus 1, Myotis
196 ricketti herpesvirus 2, and Pipistrellus nathusii rhadinovirus 1 [10, 28, 29].

However, the four MgGHVs identified here are clearly distinct from the other reported gammaherpesviruses detected in bats.

In the study, we noted the existence of two different *gB* sequences in the DNA sample positive for MgGHV_IFB11-41. The first *gB* sequence was designated MgGHV_IFB11-41_a (LC268980), and was detected with nested PCR screening for *gB*. However, the amplicon from the subsequent LD-PCR, using a gene-specific primer targeting MgGHV_IFB11-41_a, generated a different *gB* sequence from MgGHV_IFB11-41_a. The existence of these two sequences was reproducibly confirmed with PCR. In addition to the comparative phylogenetic analysis of the partial *DPOL* and *gB* sequences, this finding also suggests the presence of a coinfection with two different MgGHVs in a single fruit bat.

To investigate possible coinfections with MgGHV and MgBHV, we performed another nested PCR to screen for *gB* in the same samples in which the MgBHV sequences was detected. A gammaherpesviral *gB* sequence was detected in one (MgGHV_IFB11-45; LC268981) of the four samples. We also detected a coinfection with MgGHV and FBAHV when we amplified a partial *DPOL* sequence (MgGHV_IFB11-32: LC268932) from a fruit bat sample from which FBAHV was isolated in our previous study [12]. These observations suggest that MgGHVs can establish coinfections with other viruses of the family *Herpesviridae*.

In the phylogenetic analysis, partial incongruence was observed between the *DPOL* and *gB* trees. We also failed to recover the long continuous *DPOL-gB* region of MgGHV with LD-PCR in 12 of the 16 bats that were positive for both the gammaherpesvirus *DPOL* and *gB* genes. This suggests that the *DPOL* and *gB*

sequences obtained from the individual bats were derived from different MgGHV origins. Coinfections with several beta- or gammaherpesviruses have also been reported in other animals [7, 30]. Considering the broad reactivity of the degenerate primers used in the present study, the coexistence of different herpesviruses might be a obstacle to the effective use of the LD-PCR approach [16].

In this study, we detected 67 partial *DPOL* sequences from MgGHVs, and all of them were phylogenetically related to previously reported gammaherpesviruses detected in *Pteropus* bats. The sequences of MgGHV Group-3 shared 96%-100% identity with those of PgHV-3, and those of MgGHV Group-4 shared 99% identity with those of PgHV-2, even though the sequences were detected in different bat species. This suggests that these groups of gammaherpesviruses are transmitted independently of the host species. This hypothesis is also supported by another finding in the phylogenetic analysis of MgGHV Group-1. In this group, identical partial MgGHV DPOL sequences were detected in different bat species, *Pteropus* sp. and *Acerodon celebensis* as summarized in Table S1. It has also been reported that spillover events of alpha- and gammaherpesviruses beyond the species in the past [26, 31, 32]. It is possible that these groups of gammaherpesviruses are frequently transmitted between species and are maintained in a broad range of bat species inhabiting the Asian region.

The MgGHV Group-7 nucleotide sequence was identical to that of Pteropine herpesvirus 2, and both sequences were detected in *Pteropus vampyrus,* as shown in Fig. 2(a) and Table S1. Detailed information about the bat in which the

243 Pteropine herpesvirus 2 sequence was detected is not available, so it is unclear
244 whether this bat was imported from an Asian country or was born at the captive
245 breeding facility in the United States [22]. Escalera-Zamudio, *et al*. suggested that
246 the existence of endogenous gammaherpesviruses in the bat genome was
247 unlikely [31]. Therefore, we infer that *Pteropus vampyrus* is susceptible to
248 infection with this group of gammaherpesviruses.
249 In summary, using PCR screening, we detected genomic fragments of four
250 novel betaherpesviruses and 67 novel gammaherpesviruses, designated
251 MgBHVs and MgGHVs, respectively, in fruit bats inhabiting Indonesia. Our results
252 demonstrate the geographic distribution and genetic diversity of the MgGHVs in
253 these bat populations. The LD-PCR approach allowed us to determine a relatively
254 long region of the MgGHV genomic sequence and to infer a deep phylogeny for
255 the identified MgGHVs. Our results also suggest that coinfections with MgGHVs
256 and FBAHV and with MgBHV and defferent MgGHVs occur, and confirm the
257 interspecies transmission of MgGHV Group-1, -3 and -4. This study contribute to
258 our current knowledge of the herpesviruses in the bats of Indonesia, and the
259 phylogenetic diversity of the gammaherpesviruses.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

All experiments involving animals in Indonesia were performed in accordance with the ethical guidelines of the Animal Care and Use Committee of the Animal Teaching Hospital, Bogor Agricultural University, which are based on the Guide for the Care and Use of Laboratory Animals (7th and 8th editions) by the National Research Council of the National Academies, and the Guideline on the Care and Use of Animals for Scientific Purposes by National Advisory Committee for Laboratory Animal Research. The protocol was approved by the Animal Care and Use Committee of the Veterinary Teaching Hospital, Bogor Agricultural University (permit number 05-2010 RSHP-IPB).

All of the bat samples were collected with the permission of the Directorate General of Livestock and Animal Health Services, Ministry of Agriculture, Republic of Indonesia. The samples were stored at -80°C and have been used previously to screen for other viruses.

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Table 1. Results of PCR screening for gammaherpesviruses

	Sampling location	DPOL gene	gB gene
Pteropus vampyrus	Lima Puluh Kota	11/20	3/11
	Magelang	2/20	1/2
	Panjalu	10/26	1/10
	Surabaya	2/3	0/2
Subtotal		25/69	5/25
Pteropus sp.	Paguyaman	20/35	9/20
	Popayato	3/4	0/3
	Sidrap	6/15	0/6
	Soppeng	5/7	1/5
Subtotal		34/61	10/34
Dobsonia moluccensis	Paguyaman	6/17	2/6
Acerodon celebensis	Paguyaman	2/36	0/2
Total		67/183	17/67

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Fig. 1. Geographic information on the numbers and species of captured bats.

In total, 183 bats were captured in the Lima Puluh Kota, Magelang, Panjalu, Paguyaman, Popayato, Sidrap, Soppeng and Surabaya regions of Indonesia between 2010 and 2014. The bats were classified as four species: *Pteropus vampyrus*, *Pteropus* sp., *Dobsonia moluccensis,* and *Acerodon celebensis*.

Fig. 2. Phylogenetic analysis of partial *DPOL*, *gB* and *gB-DPOL* nucleotide sequences of MgGHV.

Phylogenetic trees of partial gammaherpesvirus (a) *DPOL*, (b) *gB* and (c) *gB-DPOL* nucleotide sequences were constructed by Maximum likelihood phylogenetic analyses in the MEGA7 program. The 67 partial MgGHV *DPOL* sequences (172 bp in length) were used for the analysis and clustered into seven groups (MgGHV Group-1 to -7) when the entire tree was visualized. When the 18 partial MgGHV *gB* sequences (417-456 bp in length) were analyzed, they clustered into six groups (MgGHV Group-A to -F). Four MgGHV *gB-DPOL* sequences (3,351-3,544 bp in length) were analyzed. All the MgGHV sequences are highlighted in gray. The bootstrap values obtained after 1,000 replicates are indicated at the major tree roots. The scale bar represents a distance of 0.1 bp of substitutions per site. The sequences detected in bats are indicated with asterisks.