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LETTER TO THE EDITOR

Bats as another potential source of murine gammaherpesvirus 68 (MHV-68) in nature

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Murine gammaherpesvirus 68 (MHV-68) is a natural pathogen of free-living murid rodents. In 1976, MHV-68 was isolated from a bank vole *Clethrionomys glareolus / Myodes glareolus* captured near Bratislava, Slovakia (*1*). Based on molecular analysis, the virus was classified to the species *Murid herpesvirus 4* (MuHV-4), the genus *Rhadinovirus*, the subfamily *Gammaherpesvirinae*, the family *Herpesviridae* and the order *Herpesvirales* (*2*).

Genome of this virus was sequenced by Virgin in 1997 (*3*). MHV-68 is an accepted animal model for the investigation of pathogenesis, oncogenesis and immunology of human oncogenic gammaherpesviruses (*4, 5*). Based on the recent ecological studies it is known that this virus may spread from its reservoir wild animals to other animal species in the same biotope as well as to livestock and household animals (*6*). The role of individual animal species in MHV-68 infection is not known. It is widely known that host-switching of a virus can have fatal consequences for the new host. Presence of serum antibodies to MHV-68 was detected in various hosts from wild reservoir (wood mouse, bank vole, field vole, yellow-necked mouse, wild mouse) or non-reservoir animals (wild boar, red fox, fallow deer, red deer, European roe deer, hare), to farm, domestic and household animals (goat, horse, cattle, dog, cat, wild house mouse), to humans (laboratory personnel working with the virus, hunters, people coming into contact with forest animals) and vectors (ticks) (*6*). Antibodies were assayed by virus neutralization assay, complement fixation test or ELISA. The presence of viral DNA in some of the samples was also confirmed by PCR.

Bats are intensively studied animals since they have been confirmed as a reservoir for many viruses, such as rabies virus or tick-borne encephalitis virus. These viruses are potentially dangerous to humans. Viruses detected in bats can also cause severe viral infection in the human population, often with fatal consequences, e.g. SARS coronavirus, MERS coronavirus, Ebola virus, Marburg virus, Hendra virus, Nipah virus. Currenty, there is only limited information on herpesviruses and bats, and while all three subfamilies of herpesviruses have been detected in bats around the world, their biology is not well studied (*7, 8, 9*).

The aim of presented study was to look for the presence of MHV-68 in blood samples from bats using serological and direct detection methods.

MHV-68 is a lymphotropic virus attacking in particular lymphoid tissue and B-cells, which results in a life-long latency. It has similar properties as important human gammaherpesviruses, e.g. Epstein-Barr virus, and, in case of weakened

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Abbreviations: MHV-68 = murine gammaherpesvirus 68

Table 1. Results of virus neutralization assay

Dilution of serum	Sample No. 4 (*B. barbastellus*)	Sample No. 5 (*M. blythii*)	Sample No. 6 (*M. blythii*)	Sample No. 10 (*M. blythii*)	Sample No. 12 (*M. blythii*)
1:4	–	–	–	–	–
1:8	–	–	–	+/-	–
1:16	+	–	+	+	+
1:32	+	+/-	+	+	+
1:64	+	+	+	+	+
1:128	+	+	+	+	+

(+) cytophatic effect on Vero cells, (–) inhibition of cytophatic effect on Vero cells.

immune system, reactivation of the virus accompanied by the development of various malignancies can occur.

Detection of MHV-68 in bats would not only expand our knowledge of the ecology and epidemiology of the virus, but it could also help to clarify the biology of herpesviruses in bats.

Serum samples from 18 bats of three different species (*Barbastella barbastellus, Myotis blythii, Myotis myotis*) captured in Muránska Planina, Slovakia, were tested by virus neutralization assay. Virus-neutralizing antibodies against MHV-68 were detected in four samples from the lesser mouse-eared bats (*M. blythii*) and in one sample from the western barbastelle (*B. barbastellus*). Antibody titers varied from 8 to 32 (Table 1). Virus-neutralization assay was performed with 2-fold dilutions of heat-inactivated bat sera. For neutralization, 1000 PFU of MHV-68 was added. The virus-serum mixtures were incubated at 37°C for 30 min and then inoculated onto Vero cell monolayers. The last dilution of serum that was able to prevent virus infection in cell culture was considered as the titer of virus-neutralizing antibodies.

Since bats are among the smallest mammals, obtaining blood samples is difficult. In order to avoid injury to the animal, only small amounts of blood can be obtained. Therefore, it was not possible to collect blood into two tubes (for whole blood and for serum separation). In our study, after sera separation and collection, the remaining clots were saved and later tested for the presence of the virus by nested PCR. Total genomic DNA was extracted using DNeasy Blood & Tissue Kit (QIAGEN) and screened for the presence of virus DNA by nested PCR targeting the ORF50 gene of MHV-68. Reactions were performed in a 50 µl volume using the GoTaq® G2 Flexi DNA polymerase protocol (Promega) with additional 2.5 mmol/l $MgCl_2$. For the detection of MHV-68 virus DNA in bat blood, two sets of primers specific for ORF 50 gene (ORF50 F1: 5'-CCACCTGATCAAATATGCCA-3', ORF50 R1: 5'-TGTGGGTTTCTTGTTTGGAC-3' and ORF50 F2: 5'-TGGCATATCCAGAGAAGTTGAG-3', ORF50 R2: 5'-TGGGAGTAGGTATGTAGCTCTG-3') were used. PCR program for the first-outer PCR was as follows: 35 cycles of 95°C for 35 s, 57°C for 35 s, and 72°C for 1 min, followed by extension at 72°C for 7 min. The second round of PCR was performed using 2 µl of the first-round PCR product as a template and the following PCR program: 40 cycles of 95°C for 40 s, 57°C for 40 s, and 72°C for 40 s, concluded with 72°C for 10 min. The sample with the highest virus-neutralizing antibody titer was found to be MHV-68 positive in nested PCR.

In addition to bats from Slovakia, we tested 20 blood samples from bats originating from Russia, Bulgaria, Poland and Ukraine for the presence of MHV-68. Total genomic DNA was extracted from bat blood cells using the High Pure PCR Template Preparation Kit (Roche) and used as a template in nested PCR targeting the ORF50 gene of MHV-68. One of the blood samples originating from the noctule bat (*Nyctalus noctula*) captured in Kharkiv, Ukraine, was positive in PCR reaction. Nested PCR product was purified using the Wizard SV Gel and PCR Clean-up System (Promega) according to the manufacturer's instructions. It was sequenced using both inner primers for the ORF50 gene-specific reaction using a commercial sequencing service. The sequence was aligned and compared with the ORF50 sequence of MHV-68 strain WUMS (Acc. No. U97553.2). Sequencing results revealed 100% homology with the ORF50 gene of MHV-68.

This is the first time that the presence of MHV-68 was confirmed in the territory of Ukraine, thereby expanding the area of its occurrence. Our results suggest that bats are another species of animals, which can serve as a reservoir of MHV-68 and can play an important role in ecology of this virus.

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