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# First evidence of murid gammaherpesvirus 4 (MHV-68) virus in the blood of oncological patients

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Murid gammaherpesvirus 4 (prototype strain MHV-68) is a natural pathogen of murid rodents that is able to transform lymphocytes and induce lymphoproliferative diseases and tumor transformation in their host cells. Considering that this virus can infect and multiply in various human cell lines, we think it could also play an important role in the pathogenesis of some human types of cancer. We investigated and confirmed the presence of the replication and transcription activator rta (ORF 50) from MHV-68 virus in four peripheral blood samples from patients with lymphomas and gliomas.

KEYWORDS

**lymphoma, glioblastoma, MHV-68, murine gammaherpes virus 4, Epstein-Barr virus**

## Introduction

The first mention of the murid gammaherpesvirus MHV-68 dates to 1980, when several of its strains were isolated (MHV-60, MHV-72, MHV-76, MHV-78, and MHV-Šumava) from *Apodemus flavicollis* and *Myodes glareolus* in Slovakia and the Czech Republic (Blaskovic et al., 1980; Mistrikova and Blaškovič, 1985). A characteristic feature of murid gammaherpesvirus MHV-68, Saimiriine gammaherpesvirus 2 (herpes virus Saimiri, HVS), Human gammaherpesvirus 8 (Kaposi's sarcoma-associated herpesvirus, KSHV), and Human gammaherpesvirus 4 (Epstein-Barr virus, EBV) is their ability to transform lymphocytes and cause lymphoproliferative and oncological diseases (lymphomas). This ability was observed both in natural hosts and in experimental conditions (Jha et al., 2016; Fickenscher and Fleckenstein, 2001; Čipková-Jarčušková et al., 2013). The course of murid gammaherpesvirus MHV-68 infection is similar to human infectious mononucleosis caused by EBV virus infection

(Tripp et al., 1997). MHV-68 is closely related to these gammaherpesviruses. The isolate Šumava undergoes a lymphoproliferative phase resembling EBV-induced infectious mononucleosis. At late stages following infection, a leukemia-like syndrome in mice developed, accompanied by significant splenomegaly, leukocytosis, and a high percentage of atypical lymphocytes in the peripheral blood (Mistríková J. et al., 2004). This brings further evidence for the lymphoproliferative effect of MHV-68 and points to the analogic course of MHV and EBV infections. Genome analysis of MHV-68 confirmed that its genome shows high homology with KSHV and EBV viruses. The genome contains 118,237 bp and encodes at least 80 genes; 63 of them are homologous to KSHV and HVS open readings frames (ORF-4 complement regulatory protein; ORF-72 D-type cyclin; and ORF-74 G-interleukin 8 receptor). MHV-68 is a model virus for studying the pathogenesis of human gammaherpesvirus infections that contribute to malignity developments, like Burkitt lymphoma, Hodgkin's disease, or Kaposi sarcoma. (Mistríková and Briestenská, 2020). Unfortunately, the lack of cell lines to support efficient *de novo* productive infection and restricted EBV and KSHV host ranges make it difficult to explore certain important biological questions. Murid gammaherpesvirus 4 can establish *de novo* lytic infection in a variety of cell lines and is also able to infect laboratory mice, offering an ideal model to study various aspects of gammaherpesvirus infection (Mistríková et al., 2004). This work aimed to find out whether the MHV-68 virus is present in some types of lymphomas and gliomas.

# Methods

Peripheral blood samples from patients with glioblastoma multiforme or lymphoma were collected based on the permission of the Ethics Committee of University Hospital, St Michaels Hospital NsM1-55/2020 by the principles of the Declaration of Helsinki.

## Blood samples

Peripheral blood samples were collected from two patients with glioblastoma multiforme and three patients with newly diagnosed lymphoma. One sample was from a patient with non-Hodgkin lymphoma/small lymphocytic lymphoma (B-NHL/SLL), one from a patient with B-chronic lymphocytic leukemia/small lymphocytic lymphoma (B-CLL/SLL), and one from a patient with non-Hodgkin lymphoma/not otherwise specified (B-NHL/NOS). As a negative control, we used blood samples from healthy individuals (four samples). Blood samples (5 mL) from patients were collected in Vacutainer K3 EDTA tubes, of which 500 µL was used for DNA isolation and the rest was stored.

## Genomic DNA isolation

DNA from human blood was isolated using a DNeasy® Blood and TissueKit (QUIAGEN Group). DNA concentration was quantified spectrophotometrically (NanoDrop 2000c Spectrophotometer, Thermo Scientific) and stored at −20°C until use.

## Detection of ORF 50 from MHV-68 genomic DNA with nested PCR

The presence of MHV-68 was confirmed by nested PCR in a sample of isolated genomic DNA. For the detection, two sets of primers specific for ORF 50 gene (ORF50 F1: 5′-CCACCTGATCAA ATATGCCA-3′, ORF50 R1: 5′-TGTGGGTTTCTTGTTTGGAC-3′and ORF50 F2: 5′-TGGCATATCCAGAGAAGTTGAG-3′, ORF50 R2: 5′-TGGGAGTAG GTATGTAGCTCTG-3′) were used (Briestenská et al., 2018; Kabát et al., 2021). Primers were designed from the ORF50 (transcriptional activator) sequence of the MHV-68 virus (NCBI reference number: NC_001826.2, positions 67979-68948).

The 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 in *Biometra's T-personal* Thermal Cycler (Biometra, Germany) (Briestenská et al., 2018; Kabát et al., 2021). The PCR product was evaluated on a 1.5% agarose gel (Supplementary Figure S1) using ethidium bromide (0.001% w/v, in water).

## Isolation of total RNA from human peripheral blood

Only PCR-positive blood samples and control samples were processed. For total RNA isolation we used 200 µL of blood. Total RNA from human blood was isolated using TRI-Reagent (Sigma-Aldrich) following the manufacturer's instructions. Prior to testing, the total amount of all RNAs (1.9–3.6 µg) was treated with DNase I (RNase-free) (ThermoScientific, United States), 1U DNase I per 1 µg RNA, in the presence of 100 mM Tris-HCl (pH 7.5 at 25°C), 25 mM MgCl2, and 1 mM CaCl2 reaction buffer for 60min. The treatment was terminated by the addition of 1 µL 50 mM EDTA per 1 µg RNA and heat-inactivation at 65°C for 15min. RNA concentration was quantified spectrophotometrically (NanoDrop 2000c Spectrophotometer, Thermo Scientific) and stored at −80°C until use.

### Reverse transcription-polymerase chain reaction (RT-PCR)

The prepared RNA was used as a template for reverse transcription with a First-Strand cDNA Synthesis kit (ThermoScientific, United States). Following the manufacturer's instructions, 300–500 ng of total RNA was reverse-transcribed into first-strand cDNA, using Random Hexamer Primers. The products of the first strand cDNA synthesis were used directly in PCR.

### Detection of ORF 50 from MHV-68 virus from prepared cDNA

Five hundred nanograms of cDNA in 0.4 μL was used as template in a 20-μL reaction mixture for PCR, using Phusion Hot Start II DNA Polymerase (ThermoScientific, United States), with the following primers: ORF50 F2: 5′-TGGCATATCCAGAGA AGTTGAG-3′, ORF50 R2: 5′-TGGGAGTAGGTATGTAGC TCTG-3'. This reaction amplified a 580 bp long fragment of the ORF50 gene. This PCR procedure was performed with 35 cycles of 98°C for 30 s, 57°C for 30 s, and 72°C for 30 s, followed by an extension at 72°C for 10 min in a Master cycler personal (Eppendorf, Germany). The PCR product was evaluated on a 1% agarose gel (Supplementary Figure S2) using ethidium bromide (0.001% w/v, in water).

### PCR product purification

DNA fragments of interest were excised in a minimal volume of agarose and purified to remove excess nucleotides and primers using Wizard® SV Gel and PCR Clean-Up System (Promega, USA), according to the manufacturer's instructions. Purified PCR-product concentration was again quantified spectrophotometrically.

### Sequencing

Sequencing was carried out by the commercial company K-Trade (Slovakia).

### Avoiding sample contamination

Contamination of blood samples during our experiments was avoided by separating all laboratories. We recognized the hazards of potential contamination; by using special precautions, we avoided the possibilities of contamination. We used 'clean' workspaces and checked the PCR reagents regularly using no-template control/negative controls with the reagents.

In the present work, all molecular biology experiments were carried out in separate laboratories. Work with live animals in these laboratories was strictly forbidden.

The animal facility is located outside the main building; it has separate laboratories for dissections. The biological samples are processed in separate laboratories. Nucleic acids are isolated in cleanrooms, where there are separate boxes and laminar flow, with each type of nucleic acid being processed in a different area.

The mixing of reagents for the Master mix is carried out in a completely different area, which is away from the laboratories where the organs are processed and from the nucleic acid isolation areas.

We routinely work with mouse cells, and in our experiments with the MHV-68 virus, we used genomic DNA from these cells, which was regularly checked for the presence of the virus (negative control). In the present work, besides the mentioned precautions, each group of workers who prepared PCR reactions had their reagents, primers, DNA, and RNA-free plasticware and worked in special laboratories with PCR boxes designed only for preparing PCR reactions. In our experiments, we did not use FSB, mouse DNA, or mouse cells.

## Results

Using nested PCR reaction, we demonstrated the presence of ORF50 from the MHV-68 virus in four samples of genomic DNA from human peripheral blood: in blood from two patients with glioblastoma, one with B-chronic lymphocytic leukemia/small lymphocytic lymphoma, and one with non-Hodgkin lymphoma/small lymphocytic lymphoma. PCR fragments of all four positive samples were sequenced to confirm the presence of the ORF50 gene. The obtained sequence of PCR fragment showed 100% homology with the ORF 50 of MHV-68 (rta -replication and transcription activator), which is specific only for this virus and has 85% homology only with wood mouse herpesvirus replication and transcription activator.

Total RNA was isolated from the blood samples in which the presence of ORF50 was confirmed, and cDNA was subsequently prepared. The quality of the prepared cDNA was confirmed by the PCR reaction (Supplementary Figure S3) of housekeeping genes (GAPDH and β-actin).

In one of the four positive blood samples, the presence of ORF 50 from MHV-68 in the genomic DNA was confirmed, and its transcript was also detected. The presence of this transcript was confirmed in blood from a patient with lymphoma.

## Discussion

Human gammaherpesvirus 4 (Epstein-Barr virus) and human gammaherpesvirus 8 (Kaposi's sarcoma-associated herpesvirus) are important human pathogens associated with

lymphoproliferative diseases and several types of human tumors (Epstein et al., 1964; Chang et al., 1994). MHV-68 is closely related to these two viruses (Macket et al., 1997). It is used as a model laboratory virus for the studies of pathogenesis of the human gammaherpesvirus infections contributing to malignity developments like Burkitt lymphoma, Hodgkin's disease, and/or Kaposi sarcoma. MHV-68 and all its strains are capable of productive infection in more than twenty cell lines originating from different organisms (rabbit, hamster, chick, mouse, mink, guinea pig, monkey, and human), especially primary and diploid embryonic cells (Čipková-Jarčušková et al., 2013). MHV-68 is able to infect and induce a productive infection in several human cell lines: in 293T cell line (a stable clone derivative of the human embryonic kidney HEK 293 cell line); in a cell line of human amniotic cells (Am-57); in the LEP cell line derived from human embryonal lungs; and in HeLa cells (Čipková-Jarčušková et al., 2013).

EBV and KSHV viruses are associated with lymphoproliferative diseases and several human tumors. The high homology of the MHV-68 virus with the human gammaherpesviruses EBV and KSHV and its ability to replicate in human cells suggests that this virus could also play an important role in the oncogenesis of human tumors. Human gammaherpesvirus infections of the central nervous system have been also linked to various neurological diseases. After infection of mice with the MHV-68 virus, it was proven that the virus may persist inside and outside the central nervous system once it gains access to it (Kang et al., 2021).

In this work, we confirmed the presence of ORF 50 from the MHV-68 virus using a nested PCR reaction in patients with different types of tumors (glioblastoma and lymphoma). The presence of the ORF 50 gene (rta - replication and transcription activator) from the MHV-68 virus was confirmed in DNA isolated from two glioblastomas and two lymphomas. In one of these positive samples, the presence of ORF50 transcripts was also confirmed in total RNA isolated from one lymphoma. These preliminary findings suggest that the MHV-68 virus can be present in the peripheral blood of patients affected by some types of oncological diseases. Future research should focus on the role of the MHV-68 virus in the pathogenesis of human tumor development.

## Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: https://www.ncbi.nlm.nih.gov/nuccore/NC_001826.2.

## Ethics statement

The studies involving humans were approved by Peripheral blood samples from patients with glioblastoma multiforme were collected based on the permission of the Ethics Committee of University Hospital, St Michaels Hospital NsM1-55/2020 by the principles of the Declaration of Helsinki. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin because Personal data of particitant were not published in the manuscript. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

## Author contributions

IŠ and PK contributed to the conception and design of the study, JD, MM, and DL carried out laboratory work, MB, PB, JJ, and TŽ provided samples for analysis. PK wrote the first draft of the manuscript and provided samples for analysis. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/av.2025.14168/full#supplementary-material

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