Folia Microbiologica
https://doi.org/10.1007/s12223-018-0586-3

SHORT COMMUNICATION

First detection of murine herpesvirus 68 in adult *Ixodes ricinus* ticks

Marcela Kúdelová[1] · Monika Jánošová[2] · Petra Belvončíková[1]

Received: 3 July 2017 / Accepted: 12 January 2018

Abstract
Murine herpesvirus 68 (MHV-68) is a natural pathogen that infects murid rodents, which serves as hosts for *Ixodes ricinus* ticks. For the first time, MHV-68 was detected in immature *I. ricinus* ticks feeding on *Lacerta viridis* lizards trapped in Slovakia, which supports the idea that ticks can acquire the virus from feeding on infected hosts. The recent discovery of MHV-68 infection and MHV-68 M3 gene transcripts in *Dermacentor reticulatus* ticks collected in Slovakia also supports this suggestion. Here, for the first time, we report MHV-68 infection, which was detected by nested PCR, in *I. ricinus* adults collected from the vegetation, and the viral load in infected ticks was determined by quantitative PCR. The viral incidence in ticks was 38.1% (21/55), and the viral load varied from 1.5×10^3 to 2.85×10^4 genome copies per tick. These results suggest that the *I. ricinus* ticks became infected with MHV-68 from biting infected rodents; thus, *I. ricinus* ticks may play a role in the spread of this virus in nature.

Keywords Murine herpesvirus 68 · *Ixodes ricinus* · MHV-68 load · qPCR · Slovakia

Introduction

The most extensively characterized viruses that have rodent hosts in the family Muridae are the members of the family Herpesviridae. Murine gammaherpesvirus 68 (abbreviated as MHV-68 or γHV68; species Murid herpesvirus 4 (MuHV-4)), classified in the genus Rhadinovirus of the subfamily *Gammaherpesvirinae* (Virgin et al. 1997), was originally isolated from bank voles (*Myodes glareolus*) (Blaškovič et al. 1980). Epidemiological surveys in Europe have since identified several other gammaherpes viruses in free-living rodents (Ehlers et al. 2007; Hughes et al. 2010). Among murid rodents, MHV-68 is transmitted via intranasal routes and through host body fluids, such as saliva, urine, tears, and breast milk (Rašlová et al. 2001). After the clearing of an acute respiratory infection in the host, MHV-68 spreads via the host's bloodstream throughout the body and, like other gammaherpesviruses, causes a life-long latent infection in host B-lymphocytes. During latency, virus reactivation may occur, resulting in repeated lytic infections and the further spread of the virus (Rajčáni et al. 1985; Rajčáni and Kúdelová 2007). Wild rodents display MHV-68 infection along with numerous pathogens acquired from ticks. In Europe, there are two important hard tick species, *Dermacentor* and *Ixodes* (Acari:Ixodidae), which both act as arthropod reservoirs for a series of zoonotic pathogens affecting wild and domestic animals and humans. These ticks are important arthropod vectors for pathogens, such as bacteria (e.g., *Rickettsia* spp., *Coxiella burnetii*, *Anaplasma phagocytophilum*, *Ehrlichia* spp., *Borrelia burgdorferi* sensu lato, *Francisella tularensis*), protozoa (e.g., *Babesia* spp.) (Labuda and Nuttall 2004; Estrada-Peña et al. 2013; Reye et al. 2013; Baneth 2014), and viruses (e.g., tick-borne meningoencephalitis virus, Colorado tick fever virus, and Crimean-Congo hemorrhagic fever virus) (Estrada-Peña and de la Fuente 2014).

Ixodes ricinus ticks are widely distributed in Europe, including in Slovakia (Černý 1972), and they cause human and animal tick-borne diseases of medical and veterinary importance, such as tick-borne encephalitis, Lyme disease, anaplasmosis, and babesiosis. Their vectorial capacity is due to their long-term co-evolution with the pathogens that they transmit, an extended lifespan (up to years), and the long-

✉ Marcela Kúdelová
virukude@savba.sk

[1] Department of Viral Immunology, Institute of Virology, Biomedical Research Center, Slovak Academy of Sciences, Bratislava, Slovakia

[2] Department of Microbiology and Virology, Faculty of Natural Sciences, Comenius University, Bratislava, Slovakia

Published online: 19 January 2018

lasting blood feeding that they exhibit in all parasitic life stages (Pagel Van Zee et al. 2007).

The first evidence of MHV-68 in ticks was found in immature *I. ricinus* ticks infesting *Lacerta viridis* green lizards, in which 15 of 799 nymphs and larvae (1.8%) were identified as virus-positive (Ficová et al. 2011). To the best of our knowledge, data on the detection of MHV-68 in adults of *I. ricinus* and their nymphs collected on vegetation are lacking to date. In 2015, Kúdelová et al. detected MHV-68 positivity in 153 of 432 *Dermacentor reticulatus* adults collected in southwestern Slovakia near the Dunaj River. Live MHV-68 was found in the salivary glands, intestines, and ovaries, indicating that the virus is capable of replication in mammalian cells, and thus suggesting that MHV-68 is a potential arbovirus. Recently, MHV-68 was documented in *Haemaphysalis concinna* ticks with an incidence of 38.3% (18/47) and a viral load of 2.0×10^2 to 9.6×10^3 (Vrbová et al. 2016). More recently, an MHV-68 load of 2.2×10^4 to 8.6×10^6 copies of the viral genome (in 38 out of 48 questing ticks) and transcripts of the MHV-68 M3 gene (in 10 out of 11 questing ticks) were identified in field-collected *D. reticulatus* ticks, providing further evidence that MHV-68 could be capable of replication in ticks (Kúdelová et al. 2017). In this study, we assessed the occurrence of MHV-68 in *I. ricinus* adult ticks collected in Slovakia and determined the amount of MHV-68 in infected ticks using quantitative real-time PCR (qPCR).

Material and methods

Study site and tick collection

Ticks were collected near waterworks in Gabčíkovo, situated in southwestern Slovakia (47° 54′ 0″ N, 17° 35′ 0″ E; 114 m, above sea level) in the spring of 2014. The location belongs to the Podunajská rovina orographic entity, which is characterized by a lowland forest ecosystem, mainly comprised of willows and poplars. The presence of different species of ticks, amphibians, migratory and nesting birds, and mammals (such as roe deer, swine, hares, pheasants, foxes, voles, gophers, hamsters, weasels and hedgehogs) is typical of this area.

Ticks were collected by dragging a blanket over the vegetation and identified based on the species level and developmental stage. The study group of 55 *I. ricinus* adult ticks were individually transferred into 0.5 mL microcentrifuge tubes and maintained alive at 4 °C prior to examination.

DNA isolation from ticks

The DNA of all ticks was individually isolated using modified alkaline lysis method as described earlier (Kúdelová et al. 2015). As an additional negative control served DNA samples of known negative *I. ricinus* tick from a tick colony.

Detection of MHV-68 DNA in ticks by PCR

To examine the tick DNA samples for the presence of MHV-68, nested PCR was used targeting the ORF 50 gene of MHV-68 (61907 to 69373 nt in the strain WUMS genome, Acc. No. AF105037) coding for the R transactivator, a conserved gene among gammaherpesviruses. We used a method allowing to detect even one copy of the MHV-68 genome as described previously (Kúdelová et al. 2015). The sequences of the outer PCR primers employed were ORF50/F1:5′-AACTGGAACTCTTC TGTGGC-3′ and ORF50/R1:5′-GGCCGCAGACATTT AATGAC-3′, which amplified a 586-bp product. The sequences of inner primers were ORF50/F2:5′-CCCCAATGGTTCAT AAGTGG-3′ and ORF50/R2: 5′-ATCAGCACGCCATC AACATC-3′, which amplified a 382-bp product. As a positive control, either DNA of MHV-68 BAC (kindly provided by Prof. Koszinowski) (Adler et al. 2000) or virion MHV-68 DNA purified according to Rašlová et al. (2001) was used. All PCR work performed complied with generally known, strict protocols to control cross-contamination. The nested PCR products were resolved on a 1.5% agarose gel stained by Goldview Nucleic Acid Stain HGV-II (Beijing SBS Genetech). To prevent accidental leaking, the PCR samples were loaded on to the gel strictly behind the ladder. Amplicons of selected MHV-68–positive ticks were purified using the Wizard® SV Gel and/or a PCR Clean-up System (Promega, USA) according to the manufacturer's instructions and then sequenced.

Determination of viral load in ticks by qPCR

Viral genome loads in samples of virus-infected ticks were identified in triplicates by a modified qPCR method specific to ORF65 described earlier (Vrbová et al. 2016). In brief, 50-ng DNA samples were used in 20-μl PCR mixtures containing 0.5 μmol/L of each primer (ORF65F: 5′GTCAGGGC CCAGTCCGTA-3′ and ORF65R: 5′-TGGCCCTCTACCTT CTGTTGA-3′) and Luminaris Color HiGreen High ROXqPCR Master Mix to amplify a 65-bp-long fragment of MHV-68 ORF65 with StepOne Real-Time PCR System (AB System, Germany). The program of the was 40 cycles at 94, 59.2, and 72 °C, each for 15 s and one cycle at 72 °C for 5 min. Analysis of the qPCR data of all samples confirmed the positive signal. The specificity of the amplification was confirmed by melting curve analysis. Data analysis of qPCR was performed using standard curve generated from Cq data of tenfold dilutions from MHV-68 BAC DNA (from 1 to 10^6 copies) serving as a standard. Virus genome copies in samples were recalculated from the extracted tick DNA yield.

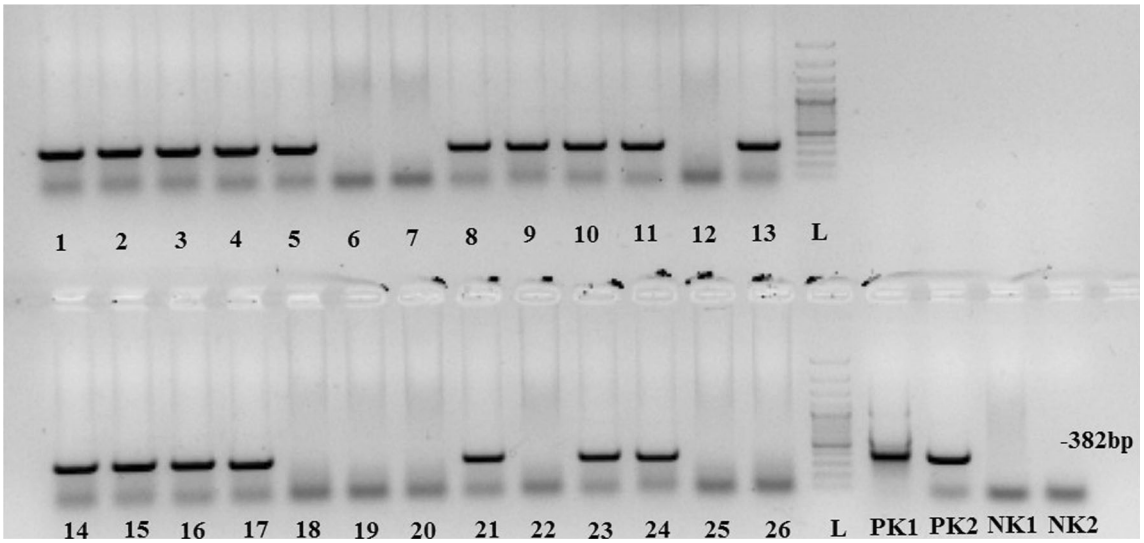

Fig. 1 Detection of MHV-68 in adult *I. ricinus* ticks collected in southwestern Slovakia in the spring of 2014 by nested PCR. *Lanes*: 1–25, tick nos. 1–25; 26, tick from a colony (negative control); L, 100-bp plus DNA ladder (Thermo Fisher); PK1, MHV-68 BAC DNA (nested PCR; positive control); NK1, no template (nested PCR negative control); PK2, MHV-68 BAC DNA (1. PCR with nested primers, positive control); NK2, no template (1. PCR with nested primers, negative control)

Sequencing analysis

The nested PCR products of 382 bp and qPCR products of 65 bp amplified from four and three DNA of randomly chosen ticks were sequenced on both strands using both relevant forward and reverse inner primer using a commercial sequencing service (BITCET) and compared with the MHV-68 ORF50 and MHV-68 ORF65 sequence (Acc. No. AF105037), respectively, by the BLAST program (www.ncbi.nlm.nih.gov/blast/).

Results and discussion

In total, 55 ticks were investigated by nested PCR for the presence of MHV-68. The tick samples that yielded nested PCR products of the expected size (382 bp) were determined to be MHV-68–positive. Sequencing of the nested PCR products of four selected virus-positive samples confirmed a nearly 100% identity with the corresponding ORF50 sequence, according to the BLAST program (www.ncbi.nlm.nih.gov/blast/) (data not shown). Twenty-one ticks carried MHV-68 DNA, representing a virus incidence of 38.1% (Fig. 1, lanes 1–5, 8–11, 13–17, 21, 23–24). These results are consistent with those found in prior studies: the incidence in a much larger group of 432 *D. reticulatus* ticks was 35.4%, and the incidence in a comparable group of 47 *H. concinna* ticks was 38.3%, demonstrating the relative independence of virus incidence in ticks with respect to study group size and locality (Kúdelová et al. 2015; Vrbová et al. 2016).

Next, the viral load of MHV-68 of all 21 MHV-68–positive samples was evaluated by qPCR, as summarized in Table 1. Sequencing of the qPCR amplicons of three selected virus-positive samples confirmed a 100% identity with the

Table 1 Determination of MHV-68 load in adult *I. ricinus* ticks by qPCR

Number of tick	MHV-68 genome copies $\times 10^4$	Number of tick	MHV-68 genome copies $\times 10^4$
1	0.29	15	0.46
2	2.36	16	0.39
3	1.99	17	0.25
4	2.85	21	1.28
5	2.15	23	0.18
8	0.23	24	0.36
9	0.31	31	1.31
10	0.25	35	0.22
11	0.25	51	0.23
13	0.15	55	0.25
14	0.28	x	x

corresponding ORF65 sequence, according to the BLAST program (www.ncbi.nlm.nih.gov/blast/) (data not shown). The viral load in *I. ricinus* adult ticks varied from 1.5×10^3 to 2.85×10^4 genome copies. In contrast, the MHV-68 load identified in *H. concinna* ticks varied from 2.2×10^2 to 9.6×10^3 genome copies (Vrbová et al. 2016). It is interesting to note that the smallest viral load identified in was ten times lower in *H. concinna* ticks. On the other hand, the highest MHV-68 load (approximately 10^4 viral genome copies) was similar in both species. More recently, an MHV-68 load of 2.2×10^4 to 8.6×10^6 copies of the viral genome was identified in field-collected *D. reticulatus* ticks (Kúdelová et al. 2017). Comparing the lowest MHV-68 load—10^2, 10^3, and 10^4 genome copies in individual tick species—*H. concinna*, *I. ricinus*, and *D. reticulatus* suggest that the amount of virus in ticks is likely to be dependent not only on the density of potential tick hosts that were infected but also on tick species. However, even 40 plaque forming units (PFU) of MHV-68 have been shown to establish a long-lived infection in experimentally infected mice (Tibbetts et al. 2003). In the first molecular study on MHV-68 in wild rodents, an approximate 34.4% prevalence of MHV-68 was detected by PCR, in the blood of free living *M. glareolus* and *Apodemus flavicollis* trapped in Slovakia (Klempa et al. 2001). It is obvious that MHV-68, due to its ability to cause persistent infection in its natural hosts and to infect a broad spectrum of cell species (mammalian, insect), fulfills at least some of the essential conditions for transmission from host to tick or vice versa (Rajčáni and Kúdelová 2003). An earlier study in the territory of Slovakia confirmed the presence of murine gammaherpesvirus-neutralizing antibodies not only in reservoir animals, such as wood mice, bank voles, field voles, yellow-necked mice, and wild mice and also in animals living in the same biotope, such as red deer (*Cervus elephus*), hare (*Lepus europeus*), and wild boar (*Sus scrofa*). Furthermore, neutralizing antibodies for this virus were found even in the sera of human subjects whose professions put them into contact with wild rodents, and in the sera of the general human population (reviewed by Wágnerová et al. (2015). Recently, live MHV-68 was identified in the salivary glands, intestine, and ovaries of free-living *D. reticulatus* ticks, and the virus was capable of replicating in mammalian cells (Kúdelová et al. 2015). An earlier report of virus-infected immature *I. ricinus* ticks (Ficová et al. 2011) showed incidences as low as 1.8%, and the finding of a virus incidence in adults >30% reported here supports the idea that ticks can acquire the virus from feeding on infected rodents such as *M. glareolus* or *A. flavicolis*. Evidence of MHV-68 in adult *I. ricinus* ticks also suggest that the virus may be vertically transmitted from nymphs to adults. However, this assumption still requires the support of experimental evidence of virus transmission within individual ticks between successive life stages. A recent finding of MHV-68 transcription in field-collected *D. reticulatus* ticks (Kúdelová et al. 2017) demonstrated that MHV-68 can replicate in these ticks, supporting the idea that ticks could act as a reservoir of murine gammaherpesvirus. It appears that murine gammaherpesvirus and ticks present another example of the following pathway: wild reservoir → vector → zoonosis. This pathway was recently identified for bats and Bartonella spp.: *B. mayotimonensis* traces were first found in bat feces, subsequently identified using PCR, and then cultivated (Veikkolainen et al. 2014). Later, a new species was described (Lilley et al. 2015) , and finally a strain that matched 100% to a patient case was found in the wild from the same area (Lilley et al. 2017). More studies are underway to verify MHV-68 transmission from tick to host and vice versa and between tick life stages in an experimental model. These studies aim to verify the proposed role of ticks in the spread of MHV-68 in nature.

Conclusions

The findings of MHV-68 in *I. ricinus* adult ticks collected on the vegetation complement earlier studies on MHV-68 infection in two other tick species, *D. reticulatus* and *H. concinna*, suggesting that MHV-68 may be a newfound pathogen (the first known among the gammaherpesviruses) detected in ticks.

Acknowledgements We thank Dr. Miroslav Slovák for collecting ticks.

Funding This work was supported by the joint grant agency of the Slovak Ministry of Education and Slovak Academy of Sciences VEGA (#2/087/17) and by the Slovak Research and Development Agency (#APVV-15-0474).

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