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A survey on murine gammaherpesvirus 68 in ticks collected in Slovakia

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Summary. – Murine gammaherpesvirus 68 (MHV-68) is a natural pathogen that infects murid rodents which serve as hosts for *Dermacentor reticulatus* and *Ixodes ricinus* ticks. For the first time, MHV-68 was detected in immature *I. ricinus* ticks feeding on lizards trapped in Slovakia. Later on, MHV-68 infection was detected in *D. reticulatus* and *Haemaphysalis concinna* ticks collected on vegetation, which supported the idea that ticks can acquire the virus from feeding on infected hosts. Here, we report MHV-68 infection, which was detected by nested PCR, in *D. reticulatus* and *I. ricinus* adult ticks and *I. ricinus* nymphs collected in five geographically isolated localities, in west, southwest, south and central Slovakia. Viral incidence in ticks was 46.7% (121/259) without considering the season, site of collection and tick species and their life stage. MHV-68 infection was detected in all five localities investigated and in both tick species. Here, for the first time, we report MHV-68 infection in *I. ricinus* nymphs collected from the vegetation. The finding of virus in ticks from five separated localities suggested that ticks became infected with MHV-68 via feeding on infected rodents; thus, this virus might be a newfound natural pathogen in ticks.

Keywords: murine gammaherpesvirus 68; Dermacentor reticulatus ticks; Ixodes ricinus ticks; nested PCR; Slovakia

Introduction

Hard ticks are obligate hematophagous ectoparasites of wild and domestic animals and humans that most notably impact global health by transmitting disease-causing pathogens, including viruses. In Europe, there are two important hard tick spp., *Dermacentor* and *Ixodes* (Acari: *Ixodidae*), which act as important arthropod vectors and reservoirs for a series of 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*., 1999; Reye *et al*., 2013; Baneth, 2014) and viruses (e.g., tick-borne meningoencephalitis virus, Colorado tick fever virus, Crimean-Congo haemorrhagic fever virus) (Estrada-Peña and de la Fuente, 2014). *I. ricinus* ticks are a widely distributed tick species in Europe, including 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. *D. reticulatus* Fabricius 1794, the three-host meadow tick that parasitizes primarily wild and domestic mammals and, infrequently, humans, is considered as the second most significant reservoir and vector of numerous pathogens causing bacterial, protozoal, rickettsial and viral diseases in its hosts. In Slovakia, the *D. reticulatus* tick had a focal distribution in Slovakia in the past (Nosek, 1972), occurring mainly in the southwest and southeast along the Morava, Danube and Latorica Rivers. Of late, *D. reticulatus* has extended its former geographical distribution in Slovakia by at least 200 km further to the North and by approximately 300 m into higher altitudes up to 520 m above sea level (Bullová *et al*., 2009).

Rodents play a role in the enzootic cycles of nonviral pathogens (*Rickettsia spp.*, *Ehrlichia spp.*, *Francisella tula-*

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Abbreviations: MHV-68 = murine gammaherpesvirus 68

rensis, and *Coxiella burnetii*) and viruses (e.g. hantaviruses, tick-borne encephalitis virus, MHV-68, and lymphocytic choriomeningitis virus). Thus, the rodents are important reservoirs for these pathogens (Burri *et al*., 2014). *Apodemus* spp. mice and *Myodes glareolus* exhibit infections with numerous tick-borne pathogens from the ticks that infest them. 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; the 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 (*M. glareolus*) (Blaškovič *et al*., 1980). Among murid rodents, MHV-68 spreads via intranasal routes and through host body fluids, such as saliva, urine, tears and breast milk (Rašlová *et al*., 2001). During acute respiratory infection in the host, it spreads from the lungs via the bloodstream to the spleen and bone marrow and via the lymphatics to the mediastinal lymph nodes. The virus causes a lifelong latent infection that may lead to lymphoproliferative disorders and tumor development. During latency, virus reactivation may occur, resulting in repeated lytic infection and further virus spread (Rajčáni *et al*., 1985; Nash *et al*., 2001, Rajčáni and Kúdelová, 2007).

In 2011, Ficová *et al*. (2011) have reported the first data on MHV-68 infection in 1.8% of immature *I. ricinus* ticks (15/799) infesting green lizards, which supports the idea that ticks can acquire the virus from feeding on infected hosts. Kúdelová *et al*. (2015) detected MHV-68 infection in approximately 40% (125/312) and 23.3% (28/120) of *D. reticulatus* adults collected in Vojka and Gabčíkovo, near the Danube River, in April 2014. Thereto, an examination of the salivary glands, intestines and ovaries of *D. reticulatus* ticks identified live MHV-68 capable of replication in mammalian cells, and thus suggesting that MHV-68 could meet some of criteria necessary for its recognition as arbovirus. However, little is known about the ecology of this virus in ticks. In this study, we assessed MHV-68 occurrence in *D. reticulatus* and *I. ricinus* ticks collected in five geographically separated localities of Slovakia using nested PCR.

Materials and Methods

Study sites. The ticks were collected in five localities of Slovakia. Four study sites, Vysoká pri Morave (near the Morava River) (48°19'50.51"N, 16°54'15.38"E), Vojka nad Dunajom (47°58´35´´N, 17°22´50´´E), Gabčíkovo (47°54'00"N, 17°35'00"E), and Komárno (47°45'48"N 18°07'42"E) are located at ~145 m, 122 m, 114 m, and 112 m above sea level, respectively. The fifth study site, near the town of Banská Štiavnica (48°27'32"N 18°53'32"), is located at ~621 m above sea level in the basin at the middle of the protected landscape area Štiavnické vrchy.

Sample collection. The study group of 259 ticks consisted of 247 adults of *D. reticulatus* (n = 230) and *I. ricinus* (n = 17) and 12 nymphs of *I. ricinus* collected over the vegetation in five localities of Slovakia in May (n = 191) and September (n = 68) 2014 (Table 1).

DNA isolation from ticks. The DNA of all ticks was individually isolated using method described earlier (Kúdelová *et al*., 2015). As negative controls DNA samples of known negative *D. reticulatus* and *I. ricinus* tick from a tick colony were used.

Detection of MHV-68 DNA in ticks by PCR. Tick DNA samples were tested for the presence of MHV-68 by nested PCR described earlier (Kúdelová *et al*., 2015), which targets the ORF 50 gene of MHV-68 (Acc. No. AF105037). The sequences of the outer PCR primers employed were ORF50/F1:5'-AACTGGAACTCTTCT GTGGC-3' and ORF50/R1:5'-GGCCGCAGACATTTAATGAC-3', which amplified a 586 bp product. The sequences of inner primers were ORF50/F2:5'-CCCCAATGGTTCATAAGTGG-3' and ORF50/ R2: 5'-ATCAGCACGCCATCAACATC-3'), which amplified a 382 bp product. As a positive control, either DNA of MHV-68 BAC or virion MHV-68 DNA purified according to Rašlová *et al.* (2001) was used. PCR mixture without template served as an additional negative control. The nested PCR products were resolved on a 1.5% agarose gel stained by Goldview nucleic acid stain HGV-II (Beijing SBS Genetech, China).

Sequencing analysis. The nested PCR products of five and four randomly chosen adult *D. reticulatus* ticks (three from Gabčíkovo and two from Komárno) and *I. ricinus* ticks (from Banská Štiavnica) were purified using the PCR Clean-up system (Promega, USA) and commercially sequenced on both strands (BITCET) and compared with the MHV-68 ORF50 sequence according to the BLAST software (www.ncbi.nlm.nih.gov/blast).

Statistical analysis. A statistical analysis was conducted using the chi-square test and Past version 2.17b software (Hammer *et al*., 2001). The analysis examined the differences in virus occurrence between *D. reticulatus* and *I. ricinus* ticks. A p-value <0.05 was considered significant.

Results

We detected 121 ticks carrying MHV-68 DNA in a total of 259 ticks collected in five localities of Slovakia. Ignoring collection season, tick species, and life stage, viral incidence in ticks was 46.7% (Table 1). Viral incidence in *D. reticulatus* adults was 30.3% in a group of 132 ticks from Gabčíkovo (Fig. 1a: lanes 2–4, 6–9, and 12–15), 53.3% in a group of 30 ticks from Komárno, 65.5% in a group of 28 ticks from Vojka nad Dunajom (data not shown), and 62.5% in a group of 40 ticks from Vysoká pri Morave (data not shown), respectively. Furthermore, viral incidence in *I. ricinus* ticks was 81.5% in a group of 17 adults from Banská Štiavnica (Fig. 1b*a*: lanes 1, 2, 4–10, 12 and Fig. 1b*b*: lanes 1–3, 5) and 25% in a group of 12 nymphs from Vysoká pri Morave (Fig. 1c: lanes 3, 5, 6),

Table 1. Detection of MHV-68 in *D. reticulatus* and *I. ricinus* ticks collected in five localities of Slovakia in the year 2014

Locality/altitude	Tick species	Time of collection	Number of MHV-68 positive/tested ticks	Virus incidence (%)
Vysoká pri Morave/145 m	*D. reticulatus*[a]	September	25/40	62.5
Vysoká pri Morave/145 m	*I. ricinus*[sN]	May	3/12	25.0
Vojka nad Dunajom/122 m	*D. reticulatus*[a]	October	23/28	82.1
Gabčíkovo/112 m	*D. reticulatus*[s]	May	40/132	30.3
Komárno /114 m	*D. reticulatus*[s]	April	16/30	53.3
Banská Štiavnica/621 m	*I. ricinus*[s]	April	14/17	82.3
Total			121/259	46.7

Notes: [s]spring, [a]autumn, [N]nymph.

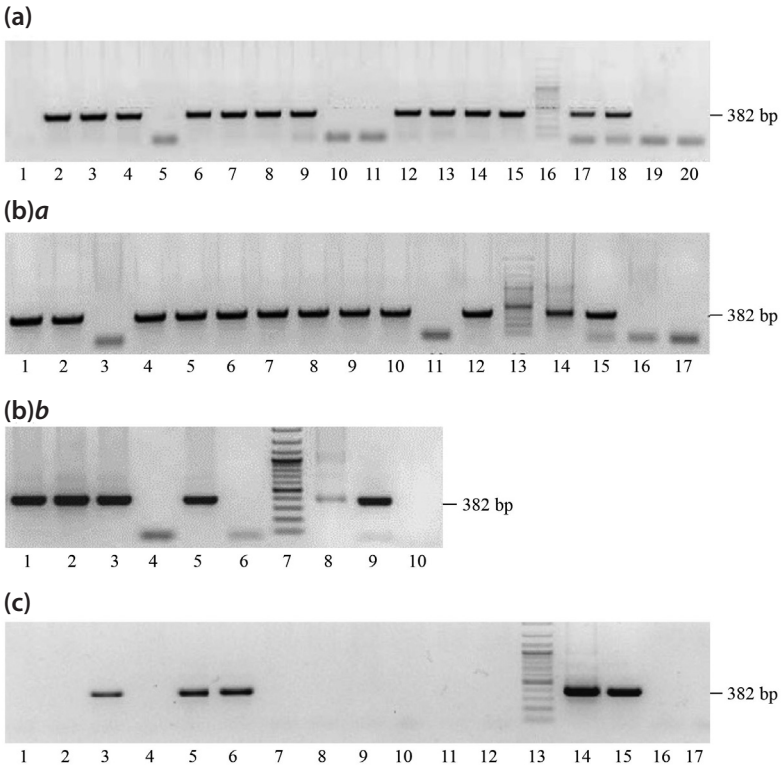

Fig. 1

Detection of MHV-68 in *D. reticulatus* and *I. ricinus* ticks collected in five localities of Slovakia using nested PCR

(a) Lanes 1–15: ticks Nos. 1–15; lane16: 100 bp ladder (Fermentas); lane17: MHV-68 BAC DNA (positive control), nested PCR; lane 18: MHV-68 BAC DNA (positive control), a single PCR with inner primers only; lane 19: negative control, nested PCR; lane 20: negative control, a single PCR with inner primers only; **(b)*a*** Lanes 1–12: ticks Nos. 1–12; lane 13: 100 bp ladder; lane 14: MHV-68 BAC DNA, a single PCR with inner primers only; lane 15: MHV-68 BAC DNA, nested PCR; lane16: negative control, a single PCR with inner primers only; lane 17: negative control, nested PCR; **(b)*b*** Lanes 1–5: ticks Nos. 13–17; lane 6: tick from colony; lane 7: 100 bp ladder; lane 8: MHV-68 BAC DNA, a single PCR with inner primers only; lane 9: MHV-68 BAC DNA, nested PCR; lane 10: negative control, nested PCR; **(c)** Lanes 1–12: ticks Nos. 1–12; lane 13: 100 bp ladder; lane 14: MHV-68 BAC DNA, a single PCR with inner primers only; lane 15: MHV-68 BAC DNA, nested PCR; lane 16: negative control, a single PCR with inner primers only; lane 17: negative control, nested PCR. Negative control – template replaced with sterile water.

respectively. Sequencing of the nested PCR products amplified from 5 *D. reticulatus* and 4 *I. ricinus* adults revealed 100% identity with the corresponding ORF 50 sequence (data not shown).

Discussion

Hard ticks *D. reticulatus* and *I. ricinus* often feed on small murid rodents, mainly of *Apodemus* spp. mice and *M. glareolus*, from which MHV-68 was originally isolated. Due to an ability to reactivate from latent infection, MHV-68 can exist for a relatively long time in the blood of murid rodents. After first confirmation of MHV-68 by molecular techniques in the blood of 34.4% of *M. glareolus* and *A. flavicollis* mice trapped in Slovakia (Klempa *et al*., 2001), several following studies have confirmed MHV-68 infection in immature *I. ricinus* ticks and *D. reticulatus* and *H. concinna* adults (Ficová *et al*., 2011; Kúdelová *et al*., 2015; Vrbová *et al*., 2016). In this study, we have confirmed MHV-68 in 46.7% of *D. reticulatus* and *I. ricinus* ticks collected in five geographically separated localities of Slovakia during a single year. The difference in viral incidence between *D. reticulatus* and *I. ricinus* ticks was not significant (45.2% *vs.* 58.6%), certainly due to the disbalanced numbers of tested ticks (130 *vs.* 29) ($\chi^2 = 1.8586$, P = 0.172787 at p <0.05). For the same reason, MHV-68 incidence strongly varied (from 30% to 82.3%) in *D. reticulatus* ticks in individual localities. Comparing MHV-68 incidence in the largest group of this study, 132 *D. reticulatus* adults from Gabčíkovo (collected in May 2014), with a group of comparable size, 120 *D. reticulatus* adults (collected in Gabčíkovo in April 2014) have shown no significant difference (30.3% *vs.* 23.3%) ($\chi^2 = 1.5497$, P = 0.213175 at p <0.05) (Kúdelová *et al*., 2015). These results are consistent with the latest report on the presence of MHV-68 M3 gene transcripts in wild *D. reticulatus* ticks, supporting the idea that MHV-68 could be a newly-discovered arbovirus (Kúdelová *et al*., 2017).

Rodents are important hosts for *Ixodes spp.* ticks especially for larvae, to some extent for nymphs and adults (Bown *et al*., 2006; Zeidner *et al*., 2000). In this study, we have found a high overall 58.6% incidence of MHV-68 in a relatively small group of 29 *I. ricinus* ticks while 25% in the nymph group. It is apparent that MHV-68 incidence in *I. ricinus* ticks needs further exploring of statistically acceptable study groups. However, results are particularly interesting because *I. ricinus* ticks were collected at a relatively high altitude ~621 m above sea level (near Banská Štiavnica town). It correlates with recent data regarding the extension of areas and the shift in the altitudinal distribution limit and abundance of ticks in Europe including Slovakia (Dautel *et al*., 2006; Wielinga *et al*., 2006; Buczek *et al*., 2013; Lukáň *et al*., 2010; Hubálek and Rudolf, 2012). Finding of MHV-68 in *I. ricinus* nymphs infesting on lizards, which serve as particularly important host of immature *I. ricinus* ticks (Casher *et al*., 2002), has given rise to the hypothesis that MHV-68 might be vertically transmitted from nymphs to adults (Ficová *et al*., 2011). This study for the first time detected MHV-68 in immature *I. ricinus* ticks collected on vegetation, thus contributing to the most recent results of experimental vertical and horizontal transmission of MHV-68 between *I. ricinus* ticks and their host - mouse and *vice versa* (Hajnická *et al*., 2017). In summary, the evidence of MHV-68 in both hard tick species and in five geographically separated localities of Slovakia investigated supports the hypothesis that ticks might facilitate MHV-68 circulation in nature. It is obvious that MHV-68 incidence in ticks strongly depends on many factors from which the density of virus-bearing host population seems to be the most important. One can hypothesize that other murid/murine gammaherpesviruses found in rodents such as *A. flavicollis*, A. *sylvaticus,* and *Mus musculus* (Blasdell *et al*., 2003; Ehlers *et al*., 2007; Hughes *et al*., 2010; Loh *et al*., 2011; Knowles *et al*., 2012) might occur in ticks feeding on these hosts.

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