Article

Effectiveness regarding hantavirus detection in rodent tissue samples and urine

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Abstract: The natural hosts regarding Orthohantaviruses are rodents, soricomorphs and bats, and it is well known they may cause serious or even fatal diseases among humans worldwide. The virus is persistent among animals and it is shed via urine, saliva and feces, throughout the entirety of their lives. We aim to identify the effectiveness regarding hantavirus detection from rodent tissue samples and urine originating from naturally infected rodents. Initially, animals were trapped at five distinct locations throughout the Transdanubian region in Hungary. Lung, liver, kidney and urine samples were obtained from 163 perished animals. All organs and urine were tested using nested reverse transcriptase-polymerase chain reaction (nRT-PCR). Furthermore, sera were examined for IgG antibodies against DOBV and PUUV viruses by Western Blot assay. IgG antibodies against hantaviruses and/or nucleic acid were detected in 25 (15.3%) cases. Among *Apodemus*, *Myodes*, and *Microtus* rodent species, DOBV, PUUV, TULV were all clearly identified. The virus nucleic acid was detected most effectively from the kidney (100%), while only 55% of screened lung tissues were positive. Interestingly, only 3 out of 20 rodent urine samples were positive regarding nRT-PCR. Moreover, five rodents were seropositive without detectable virus nucleic acid from any of the tested organs.

Keywords: naturally infected, hantavirus detection, urine, rodent, tissue

1. Introduction

Orthohantaviruses (*Hantaviridae* family) are negative-sense, single stranded RNA viruses with three genome segments including the S segment (encodes the nucleoprotein), M segment (encodes glycoproteins) and the large L segment which encodes the RNA-dependent RNA polymerase [1]. Orthohantaviruses may cause serious or even fatal diseases, such as the hemorrhagic fever with renal syndrome (HFRS), caused by Hantaan (HNTV), Dobrava-Belgrade (DOBV) and Seoul (SEOV) viruses, while Puumala virus (PUUV) is the etiological agents in reference to nephropathia epidemica (NE). The mortality rates of HFRS ranging from 3 to 15% and dependent on the causative agents. In contrast, PUUV is responsible for more than 9000 infections annually, throughout Europe, with a significantly lower-case fatality rate of 0.1-0.4%. Among New World orthohantaviruses, the Sin Nombre (SNV) and Andes orthohantaviruses (ANDV) cause HCPS with an average case fatality rate at or near 40% [2–4]. Globally, 150,000-200,000 human cases of orthohantavirus infections are reported annually [5]. Hantaviruses transmitted to humans by persistently infected rodents, soricomorphs and bats indirectly via inhalation of aerosolized excreta of infected animals or directly

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through a rodent bite [6]. In Europe, the two major human pathogenic orthohantaviruses are DOBV, carried by the yellow necked mouse (*Apodemus flavicollis*), the striped field mouse (*Apodemus agrarius*), and the wood mouse (*Apodemus sylvaticus*) while PUUV are carried by the bank vole (*Myodes glareolus*) [1,7,8]. From a varied perspective, Tula virus (TULV) can be found in Europe [9,10] yet the human pathogenic nature intrinsic to its species is debated. In consideration of their natural animal hosts, these viruses do not cause disease, despite its influential characteristics upon the hosts survival and cause histopathological changes regarding infected tissues [3,11,12]. Virus infection induces IgG antibody response following 2-3 weeks and it lasts life-long throughout small mammals. However, this life-long presence of the virus in tissues and excreta is questionable.

In this study, we aim to identify the most suitable tissue in the detection of hantavirus. For this reason, various rodent tissues and urine originated from naturally infected animals were tested by molecular detection method. Additionally, it was also an important question whether naturally infected rodents are able to transmit the virus over a lengthy period of time via urine, as it was formerly hypothesized.

2. Materials and Methods

Sample collection

Rodents were trapped as part of an ecological research at five different locations throughout the Southern Transdanubian Region, between 2012-2015, and from March to October. Rodent live traps were used with quadrat sampling pattern in each trapping period. In every month, standard five-night capture occasions were carried out. The traps were checked once/twice per day depending on the trap location.

Perished animals acquired from live traps were used in our study. After the species, sex and weight determination, rodents were frozen and stored until dissection. During autopsy, internal organs such as the lung, liver, kidney were removed. Urine was taken directly from the bladder using a syringe when it was available. All samples were stored at -80° C until further analysis.

Extraction of nucleic acid, PCR amplification, Sequencing

Nearly 50 mg of lung, liver and kidney tissue samples were homogenized in 500 µl phosphate-buffered saline (1×PBS) using Minilys homogenizer (Bertin Instruments) with one glass bead (2.5-2.8 mm). Afterwards, viral nucleic acid was extracted from 200 µl centrifugated (8000×g 10 min) tissue supernatant or urine using Viral Nucleic Acid Extraction Kit II (Geneaid) in accordance with the manufacturer's recommendations. The NA elution was stored at -80 °C until analysis.

In regards to hantavirus RNA detection, we used nested reverse transcription–polymerase chain reaction (nRT-PCR) using previously published specific primers (Klempa et al. 2006). The reaction was performed using QIAGEN OneStep RT-PCR Kit (Qiagen) with the following conditions: at 50 °C, for 30 min followed by an initial denaturation at 95 °C, for 15 min and then, by 40 cycles of amplification (each cycle included a denaturation step at 94 °C for 1 min, an annealing step at 53 °C for 30 sec and an extension step at 72 °C for 1 min) and a final elongation at 72 °C for 10 min. 2 µl of the first round PCR products were amplified with inner primers under the following conditions: initial denaturation at 95 °C for 5 min, followed by 40 cycles of amplification (denaturation, 94 °C for 1 min, annealing, 52 °C for 45 sec and extension on 72 °C for 1 min) and a final elongation at 72 °C for 10 min. Second round PCR product were visualized by agarose-gel electrophoresis in 2% agarose gel stained with GR Green (Labgene Scientific).

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The amplicons from positive samples were purified by a Gel/PCR DNA Fragments Kit (Geneaid) and bi-directionally sequenced with a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems™) on an ABI Prism 310 DNA Sequencer (Applied Biosystems™). Nucleic acid sequences were identified by GenBank BLAST searches based on the most significant homology.

Serological screening by Western blot analysis

Serological screening by Western blot analysis

Rodent blood samples were screened for the presence of IgG antibodies against DOBV and PUUV by Western blot (WB) analysis. We applied recombinant DOBV and PUUV antigens, both produced in an *Escherichia coli* bacterial expression system, as previously described [13,14]. Nucleocapsid proteins (PUUV and DOBV) were loaded into the wells of Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad Laboratories). Following electrophoresis, proteins were transferred to 0.45 μm pore size nitrocellulose membranes (Bio-Rad Laboratories) using Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad Laboratories), at 0.12 A for 30 min. The membranes were painted with Ponceau S (Sigma-Aldrich) which made the proteins visible. 5% non-fat dry milk was used for blocking (Blotting-Grade Blocker, Bio-Rad Laboratories) in TRIS-buffered saline (TBS) (pH=7.5), for one hour. Rodent blood samples were diluted 1:100 in TBS (pH=7.5) containing 0.1% Bovine Serum Albumin (BSA) (Sigma-Aldrich) and 0.05% Tween® 20 (Sigma-Aldrich) and membranes were incubated for 30 min at room temperature. Following incubation, membranes were rinsed in 0.05% TBS-Tween® 20 (TBS-T) for 3×10 min. Horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dako) was used as a secondary antibody, diluted 1:800 in TBS-T containing 0.05% BSA. Next, an incubation period for 30 min at room temperature was performed. Membranes were rinsed for 10 min and three times using TBS-T and once (10 min) with TBS. Development was carried out using 3,3′- diaminobenzidine (DAB) (Bio-Rad Laboratories) in TBS in accordance with the manufacturer's recommendations.

Ethical statement

The National Inspectorate for Environment and Nature Protection (Hungary) provided an ethical statement allowing the trapping and marking of the rodents in specified nature reserve areas throughout Hungary.

3. Results

In our study, we examined various tissues and urine samples originating from 163 out of 665 trapped and perished animals. The trapping was part of an ecological study with 25,183 catch number. Rodents were categorized in seven different seven species: 22 (13.5%) A. agrarius, 64 (39.2%) A. flavicollis, 6 (3.7%) A. sylvaticus, 53 (32.5%) M. glareolus, 6 (3.7%) field voles (Microtus agrestis), 11 (6.7%) common voles (Microtus arvalis) and 1 (0.6%) European water voles (Arvicola amphibious). Out of a total of 163 rodents, hantavirus nucleic acid and/or IgG antibodies against hantaviruses was detectable in 25 cases (15.3%). In consideration of these rodents, 19 belonged to the Apodemus species, 4 to the Microtus species and 2 were *Myodes glareolus*. (Table 1.) There were 20 hantavirus positive samples acquired using nRT-PCR from at least one of the investigated organs (lung, liver and kidney). Among these 20 rodent samples, the kidney tissues were positive in each rodent (20/20; 100%), while the fewest PCR positive samples originated from lung tissue (11/20; 55%). It is very likely the virus is present in the urine for only a brief span of time, since only 3 positive urine samples were detected, whereas all 20 kidney tissues were positive regarding hantaviruses.

Table 1. Summary of hantavirus positive rodents (nRT-PCR and WB serology) (Abbreviations: AAG: Apodemus agrarius, AFL: Apodemus flavicollis, MAR: Microtus arvalis, MAG: Microtus agrestis, MGL: Myodes glareolus, nt.: not tested by serology)

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Species	Number of hantavirus positive samples based on nRT- PCR / Tested samples				Serology (IgG) Positive/Tested
	Lung	Liver	Kidney	Urine	
AAG	3/9	8/9	9/9	1/9	7/9
AFL	4/10	6/10	6/10	0/10	9/10
MAR	3/3	3/3	3/3	1/3	nt.
MAG	1/1	1/1	1/1	1/1	nt.
MGL	0/2	1/2	1/2	0/2	2/2
Total	**11/25**	**19/25**	**20/25**	**3/25**	**18/21**

In reflecting upon the serological investigations, antibodies against hantaviruses in the sera were detected in 18 cases out of 25 hantavirus positive samples. In five cases, nucleic acid could not be detected in any tested organs; however, IgG antibodies were present in the sera, meaning these rodents were exclusively seropositive. The presence of the maternal antibody can be ruled out since these individuals were adults. In another 13 cases, both hantavirus nucleic acid and IgG antibodies against hantaviruses were present. Due to the lack of detection assay, Microtus voles were not tested by any serological test (Table 2).

Table 2. Detailed molecular biological results of various organs and urine samples originated from rodents along with the serological results. (Abbreviations: AAG: Apodemus agrarius, AFL: Apodemus flavicollis, MAR: Microtus arvalis, MAG: Microtus agrestis, MGL: Myodes glareolus, nt.: not tested by serology)

	Species	Lung	Liver	Kidney	Urine	Serology
1	AAG1	Pos	Pos	Pos	-	-
2	AAG2	-	Pos	Pos	Pos	-
3	AAG3	Pos	Pos	Pos	-	Pos
4	AAG4	Pos	Pos	Pos	-	Pos
5	AAG5	-	Pos	Pos	-	Pos

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6	AAG6	-	Pos	Pos	-	Pos
7	AAG7	-	Pos	Pos	-	Pos
8	AAG8	-	Pos	Pos	-	Pos
9	AAG9	-	-	Pos	-	Pos
10	AFL1	Pos	Pos	Pos	-	-
11	AFL2	Pos	Pos	Pos	-	Pos
12	AFL3	Pos	Pos	Pos	-	Pos
13	AFL4	Pos	Pos	Pos	-	Pos
14	AFL5	-	Pos	Pos	-	Pos
15	AFL6	-	Pos	Pos	-	Pos
16	AFL7	-	-	-	-	Pos
17	AFL8	-	-	-	-	Pos
18	AFL9	-	-	-	-	Pos
19	AFL10	-	-	-	-	Pos
20	MAG	Pos	Pos	Pos	Pos	nt.
21	MAR1	Pos	Pos	Pos	-	nt.
22	MAR2	Pos	Pos	Pos	-	nt.
23	MAR3	Pos	Pos	Pos	Pos	nt.
24	MGL1	-	Pos	Pos	-	Pos
25	MGL2	-	-	-	-	Pos

No clear correlation was found among virus detection from the lung and seropositivity. In two cases, negative serological results were obtained even when the virus was clearly detectable by nRT-PCR in the lung tissue. In contrast, 13 animals with a negative nRT-PCR result obtained from the lung

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were seropositive. In case of liver and kidney tissues, a different detection rate was observed. For liver and kidney, the number of nRT-PCR positive and serology negative animals was 3 for both tissues, while the number of nRT-PCR positive and serology negative rodents was 6 for liver and 5 for kidney. Unfortunately, we could not determine when did the infection occur, and how did it develop over time, since our examination was based on samples collected strictly from perished or deceased animals.

Based on a sequencing data we identified DOBV in Apodemus mice, PUUV in *Myodes glareolus* and TULV in Microtus voles, with the greatest homology of 100%, 95% and 89%, respectively.

4. Discussion

Hantavirus infections are considered as a persistent infection in rodents. A number of studies illustrate how hantaviruses are present in various types of tissues and excreta (saliva, urine and feces) [15–20]. Few studies focused on naturally infected rodents, substantiating the premise in which there are differences in virus shedding between naturally and laboratory infected rodents [16]. We examined naturally infected, perished animals collected from box traps. For years, researchers thought the most appropriate tissues regarding hantavirus detection was limited to the lung [19–22]. In our study, out of 20 PCR positive lung tissue samples, only 11 (55%) were positive for hantaviruses, while 19 liver and all kidney tissue samples demonstrated virus nucleic acid positivity. Yanagihara et al. and Gavrilovskaya et al. found how the PUUV infection caused persistent infection among rodents, therefore, the virus antigen was detected in lung tissues for nearly a year. In contrast, the virus was undetectable in the kidney [20,23]. Our research shows contrasting results regarding the case of DOBV in *Apodemus* mice, yet, in the case of PUUV, we could not determine since two *Myodes glareolus* samples were positive, thus further investigations will be necessary. Lee et al. investigated Hantaan virus (HNTV) infected *Apodemus agrarius,* which were able to infect their cage mates via urine and saliva [19]. However, the detection of the virus in the kidney did not conclude its detection from the urine. Due to low number of positive samples, the premise may be considerably murky and, additional in vivo studies are required. Presumably, among *Apodemus* mice, the host immune system can eliminate the virus among host rodents. In a review authored by Meyer & Schmaljohn, these differences were highlighted, in which PUUV antigen persistence in lung tissues was detectable for nearly a year while HNTV antigen was detectable for just 14 days [18]. According to our results, the most suitable method for detecting hantaviruses in rodents is to first screen the animals by a serological test and then, in the case of seropositive rodents, select the liver or, more preferably, the kidney for molecular biological detection. It is clearly evident from our examinations that both organs (liver and kidney) have better detectability rates of the virus than the lung used in many surveys so far.

In this study we could not confirm, in which there is lifelong virus shedding via urine. The most appropriate tissue regarding hantavirus detection by PCR methods is the rodent kidney, however if there are detectable virus particles in the kidney, we cannot be absolutely certain if these are also in the urine. Based on our results, long-term experiments must be considered in order to gain knowledge regarding the detailed nature of the virus infection.

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Conflicts of Interest:

The authors declare no conflict of interest.

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