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Short communication

Detection of rat hepatitis E virus, but not human pathogenic hepatitis E virus genotype 1–4 infections in wild rats from Lithuania

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ABSTRACT

Rat hepatitis E virus (HEV) is an orthohepevirus which is related to other HEV found in humans and other mammals. It was first identified in Norway rats (*Rattus norvegicus)* from Germany in 2010, and later it has been detected in Black rats (*Rattus rattus)* and Norway rats from USA, China, Indonesia, Vietnam and many European countries. In this study, we describe molecular and serological investigations of Black and Norway rats trapped in Lithuania, Eastern Europe, for infections with rat HEV and human HEV genotypes 1–4. Rat HEV-specific real-time reverse transcription-PCR (RT-qPCR) analysis of rat liver samples revealed the presence of rat HEV in 9 of 109 (8.3%) samples. In contrast, a RT-qPCR specific for HEV genotypes 1–4 did not reveal any positive samples. A nested broad spectrum RT-PCR was used for a confirmation of rat HEV infection with a subsequent sequencing of the amplified rat HEV genome fragment. Phylogenetic analysis revealed a clustering of all newly identified rat HEV sequences with Norway rat-derived rat HEV sequences from Germany within the species *Orthohepevirus C*. An indirect ELISA using a yeast-expressed truncated rat HEV capsid protein variant revealed 31.2% seropositive samples indicating a high rate of rat HEV circulation in the rat population examined. In conclusion, the current investigation confirms rat HEV infections in Norway and Black rats in Lithuania, Eastern Europe, and the non-persistent nature of HEV infection.

1. Introduction

Hepatitis E virus (HEV) is a member of the family *Hepeviridae* and it contains a positive-sense, single stranded RNA genome. The species *Orthohepevirus* A includes seven HEV genotypes, which infect various mammalian hosts (Smith et al., 2014, 2016; Woo et al., 2014). HEV genotype 3 is a zoonotic virus that is found in domestic pigs, wild boars, deer and other mammal reservoirs. It mainly spreads to human through consumption of meat and meat products of infected animals (Kamar et al., 2017). HEV-like viruses were detected in chicken (species *Orthohepevirus B*), ferret, mink, fox (species *Orthohepevirus C*, genotype C2), bats (species *Orthohepevirus D*), and moose (not assigned). Rat HEV (*Orthohepevirus C*, genotype C1) was first identified in 2010 in Norway rats (*Rattus norvegicus*) from Germany (Johne et al., 2010b). Since then it has been detected in Norway rats and Black rats (*Rattus rattus*) from other European countries, USA, China, Indonesia and Vietnam (Li et al., 2011; Purcell et al., 2011; Li et al., 2013b; Wolf et al., 2013; Mulyanto et al., 2014; Widén et al., 2014; Ryll et al., 2017).

The zoonotic potential of rat HEV is still a matter of debate. There are reports of a possible rat HEV transmission to humans (Dremsek et al., 2012; Shimizu et al., 2016). Recently, an HEV investigation of zoo animals in Germany resulted in the detection of rat HEV RNA in a Syrian brown bear (*Ursus arctos syriacus*) (Spahr et al., 2017). On the other hand, Norway rats were found to be infected with zoonotic HEV

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Fig. 1. Location of the trapping sites of Black rats and Norway rats in Lithuania. Names of the districts where the rats were captured are given and the number of animals trapped is indicated in round brackets. Full circles, trapping sites with rat HEV RNA or rat HEV-specific antibody positive samples; empty circles, trapping sites where only rat HEV negative samples were collected.

Table 1
Forward (F) and reverse (R) primers and probes (P) used in RT-PCR assays.

RT–PCR assay	Primers and probes	Primer/probe sequence[1]	PCR target of HEV genome	Reference
Rat HEV specific RT–qPCR	rHEV–F	5'–TACCCGATGCCGGGCAGT–3'	5220–5293[2]	Widén et al. (2014)
	rHEV–R	5'–ATCYACATCWGGGACAGG–3'		
	rHEV–P	5'–6–FAM–AATGACAGCACAGGCACCGGCGCC-BHQ–1–3'		
HEV genotype 1–4 specific RT–qPCR	HEV1–4_F	5'–GGTGGTTTCTGGGGTGAC–3'	5286–5353[3]	Jothikumar et al. (2006), modified
	HEV1–4_R	5'–GGGTTGGTTGGATGAATATAGG–3'		
	HEV1–4_P	5'–6-FAM-TTGATTCTCAGCCCTTCGC–BHQ-1–3'		
Nested broad spectrum RT-PCR	HEV–cs	5'–TCGCGCATCACMTTYTTCCARAA–3'	3977–4446[2]	Johne et al. (2010b)
	HEV–cas	5'–GCCATGTTCCAGACDGTRTTCCA–3'		
	HEV–csn	5'–TGTGCTCTGTTTGGCCCNTGGTTYCG–3'	4082–4413[2]	
	HEV–casn	5'–CCAGGCTCACCRGARTGYTTCTTCCA–3'		

[1]D = A, G or T; M = A or C; N = A, C, G or T; R = A or G; Y = C or T; BHQ–1 – Black hole quencher; 6–FAM – 6-carboxyfluorescein.
[2]according to rat HEV strain rat/R63/DEU/2009 (GenBank: GU345042).
[3]according to HEV-3 strain wbGER27 (GenBank: FJ705359).

genotype 3 strains (Kanai et al., 2012; Lack et al., 2012).

In the current study, the frequency of rat HEV infection was investigated in Lithuanian wild rats (n = 109) using real-time RT-PCR (RT-qPCR) assays targeting rat HEV and human pathogenic HEV genotypes 1–4. A conventional RT-PCR assay was used for amplification of rat HEV sequences and their subsequent phylogenetic analysis. In addition, rat HEV-specific IgM and IgG antibodies were investigated by an in-house ELISA using yeast-expressed truncated rat HEV capsid protein (Simanavicius et al., 2018). To the best of our knowledge, this is the first molecular and serologic investigation of rat HEV in Lithuanian wild rats.

2. Methods

2.1. Collection of samples from wild rats

A total of 109 wild rats (27 Norway rats, 82 Black rats) were captured during pest control measures from January 2014 to October 2017. Rat trapping was performed using snap traps placed at various locations in Lithuania (Fig. 1) including a poultry yard and sites near human dwellings; in some of them domestic animals such as pigs, chicken or cows are kept. Characteristics of the rats such as species, time and place of capture, gender, age, body weight and dimensions were determined. Chest cavity fluid (CCF) and liver samples were collected from the animals and stored at −80 °C until testing.

2.2. RNA extraction and RT-qPCR analyses

One hundred nine rat liver samples were homogenized using tissue grinders and total RNA was extracted using GeneJET RNA Purification Kit (Thermo Fisher Scientific, Vilnius, Lithuania) following the manufacturer's specifications. A two-step RT-qPCR TaqMan system was selected for rat HEV detection and quantification in rat liver samples. Two µg of total RNA was treated with dsDNase (Thermo Fisher Scientific) and thereafter RNA was reverse-transcribed to cDNA at 42 °C for 60 min with random hexamer primers using RevertAid RT Kit (Thermo Fisher Scientific). Two µl of the cDNA was amplified in the 20 µl Maxima Probe qPCR reaction mixture (Thermo Fisher Scientific) using rat HEV specific RT-qPCR system as described previously (Widén et al., 2014). This RT-qPCR assay target the region 5220–5293 in the rat HEV rat/R63/DEU/2009 sequence. The rat HEV-specific qPCR was performed on a Rotor-Gene Q MDx instrument (Qiagen, Hilden, Germany) for 10 min at 95 °C, followed by 55 cycles for 30 s at 95 °C, 60 s at 60 °C and 30 s at 72 °C using primers and probe given in Table 1.

In addition, all rat liver samples were analyzed by two-step RT-qPCR assay specific for HEV genotypes 1–4 as described previously (Jothikumar et al., 2006) with some modifications. The target of this RT-qPCR system is a region of ORF2 conserved for HEV genotypes 1–4.

Reverse transcription reaction was carried out as described above. The qPCR amplification was performed for 10 min at 95 °C, followed by 55 cycles for 30 s at 95 °C, 60 s at 58 °C and 30 s at 72 °C using forward primer HEV1–4_F, reverse primer HEV1–4_R and probe HEV1–4_P (Table 1) in the 20 μl Maxima Probe qPCR reaction mixture.

RNA standards were synthesised for quantification of HEV RNA. Briefly, the target sequences of both RT-qPCR assays were inserted into the pTZ57R vector (Thermo Fisher Scientific). Vectors that contain a 64 bp fragment of rat HEV strain rat/R63/DEU/2009 (GenBank: GU345042) and a 70 nt fragment of HEV genotype 3 isolate wbGER27 (GenBank: FJ705359) were *in vitro* transcribed using Transcript AID T7 High Yield Transcription Kit (Thermo Fisher Scientific). After that, reaction mixture was treated with DNase I and transcripts were purified and used for optimization of RT-qPCR assays, preparation of the standard curves and viral RNA quantification. The limit of detection as determined by serial dilutions of *in vitro* transcribed RNA was one genome equivalent per PCR reaction (data not shown).

2.3. Sequence determination and HEV sequence analysis

For confirmation of the presence of rat HEV RNA in liver samples, a conserved ORF1 region was amplified *via* nested broad-spectrum PCR (NBS-RT-PCR) as described previously (Johne et al., 2010b). The PCR products were purified from a gel using GenJET Gel Purification Kit (Thermo Fisher Scientific), cloned into pJET1.2 vector using CloneJET PCR Cloning Kit (Thermo Fisher Scientific) and sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and ABI PRISM 3130xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Multiple sequence alignments were generated and consensus sequences of at least 3 parallel constructs of the same rat liver sample were created with DNASTAR Lasergene 7. These sequences were deposited in GenBank under accession numbers MH400712-MH400717. All sequences were aligned with known rat HEV sequences as well as reference sequences of other *Orthohepevirus* species. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 7 (Kumar et al., 2016). The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Bootstrap values were determined based on 2000 re-samplings of the data sets. Bootstrap values equal or over 70% were considered as realiable.

2.4. Enzyme-linked immunosorbent assay for detection of antibodies to rat HEV

The presence of rat HEV-specific IgG and IgM antibodies in CCF samples of wild rats was investigated by an indirect ELISA using truncated rat HEV capsid protein comprising amino acid residues 112–608 (Simanavicius et al., 2018). Ninety six-well microtiter PolySorp plates (Nunc, Roskilde, Denmark) were coated with 50 μl of the rat HEV antigen solution (3 μg/ml) in coating buffer (50 mM sodium carbonate, pH 9.5) by 16 h incubation at 4 °C. The plates were blocked for 1 h at room temperature (RT) with RotiBlock (Carl Roth, Karlsruhe, Germany). Then antigen-coated plates were incubated with wild rat CCF or polyclonal antibodies from laboratory Wistar rats immunized with yeast-expressed rat HEV capsid protein (Simanavicius et al., 2018) (as a positive control) and non-immunized (as a negative control) Wistar rats. Antibodies were diluted with phosphate-buffered saline (PBS)-0.1% Tween-20 (PBS-T) and incubated for 1 h at RT. After washing with PBS-T, plates were incubated with either horse radish peroxidase (HRP)-labelled goat anti-rat IgG (H + L) cross-adsorbed secondary antibody (Invitrogen, Carlsbad, CA, USA) diluted 1:5000 or HRP-labelled goat anti-rat IgM secondary antibody (Invitrogen) diluted 1:1000 in PBS-T for 1 h at RT. The enzymatic reaction was started by adding TMB (3,3′,5,5′-tetramethylbenzidine, HRP substrate, NeA-Blue, Clinical Science Products Inc., Mansfield, MA, USA) and stopped by addition of 3.6% H_2SO_4. Optical density (OD) was measured using a Multiskan™ GO microplate spectrophotometer (Thermo Fisher Scientific) and calculated as the difference between OD values at 450 nm and 620 nm. Then OD values were normalized as the sample to positive control ratios (S/Ps). The cut-off value for both IgG and IgM ELISA was calculated as the mean S/P + 2 SDs of 4 non-immunised Wistar rats.

In addition to the IgG and IgM ELISA described above, a commercially available HEV genotype 1 antigen based ELISA (Axiom HEV Ab, Axiom, Bürstadt, Germany) for detection of HEV-specific antibodies was used according to the manufacturer's protocol.

3. Results

One hundred nine wild rats were captured including 27 Norway rats and 82 Black rats. RNA samples extracted from rat liver were subjected to testing by RT-qPCR systems. Nine of 109 (8.3%) liver samples were positive in the rat HEV-specific RT-qPCR assay. The rat HEV RNA was detected in 6 Black rats and 3 Norway rats. All rat liver samples were found to be negative for HEV genotypes 1–4 as determined by RT-qPCR assay specific for HEV genotypes 1–4. Only 6 of 9 rat HEV RT-qPCR positive samples were also positive in NBS-RT-PCR. The PCR products were subjected to sequencing. Multiple sequence alignment of the newly obtained rat HEV sequences revealed 98.7%–99.4% nucleotide sequence identity to HEV genotype C1 reference strain rat/R63/DEU/2009 (GenBank: GU345042) (Johne et al., 2010a). Phylogenetic analysis showed that all newly identified rat HEV sequences, independently of the rat species, cluster together with rat HEV sequences, species *Orthohepevirus C*, genotype C1 found in Germany (Fig. 2).

To evaluate the seroprevalence of rat HEV in the trapped rats, the presence of rat HEV-specific IgM and IgG antibodies was examined by ELISA. Thirty four of 109 (31.2%) CCF samples were found to be seropositive. Thirteen (11.9%) rats were only IgM positive, 13 (11.9%) were only IgG positive and 8 (7.3%) were both IgM and IgG positive (Fig. 3). A parallel analysis of rat CCF samples with a commercial HEV-1 based ELISA showed no reactivity (data not shown). Five of 34 seropositive rats were also HEV RNA positive, representing 4.6% of total population examined. In Table 2, the observed regional distribution of both HEV RNA and seropositive rat samples are shown. No association of serological and molecular HEV detection with individual characteristics of wild rats was found (data not shown).

4. Discussion

In this study, we examined Norway rat and Black rat liver samples from Lithuania for the presence of rat HEV and other hepeviruses using RT-qPCR specific for either rat HEV (Widén et al., 2014) or HEV genotypes 1–4 (Jothikumar et al., 2006). Nine of 109 wild rat samples were found to be positive for rat HEV RNA using rat HEV-specific RT-qPCR. No positive samples were detected using HEV genotype 1–4 RT-qPCR. This finding is in line with previous investigations which demonstrated that Norway rats are not susceptible to HEV genotypes 1, 3 and 4 infection (Li et al., 2013a; Ryll et al., 2017). Furthermore, the results of the current study confirm rat HEV infections in Black rats from Europe as it was shown only once earlier (Ryll et al., 2017). The phylogenetic analysis of NBS-RT-PCR (Johne et al., 2010b) products with a selection of other HEV sequences revealed that all obtained sequences cluster together with rat HEV sequences detected in Germany. This pattern of clustering of HEV genomic sequences obtained from relatively close geographical regions was observed earlier and shows the circulation of rat HEV in local populations (Johne et al., 2012; Li et al., 2013b; Widén et al., 2014; Ryll et al., 2017).

In addition to molecular analysis, rat CCF samples were investigated by an indirect ELISA for the presence of anti-rat HEV IgM and IgG antibodies. Analysis of 109 CCF samples revealed a total of 34 samples (31.2%) positive for either IgM or IgG or both IgM and IgG antibodies against rat HEV. The level of HEV seroprevalence demonstrated in this study is slightly higher than 24.5% previously described in Germany

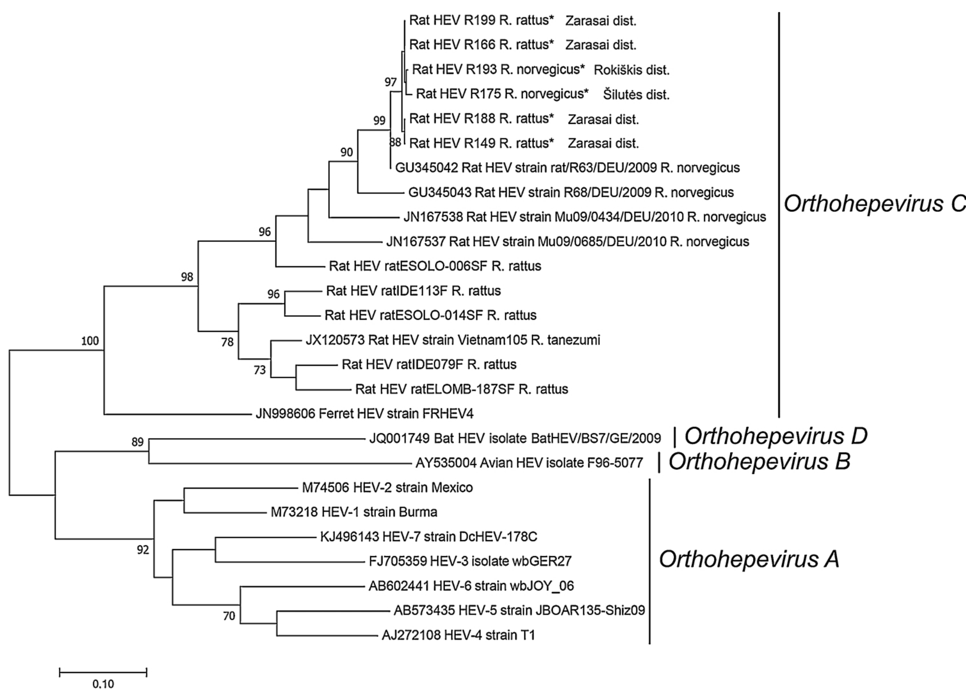

Fig. 2. Phylogenetic tree showing the relation of HEV sequences obtained from wild rats trapped in Lithuania to various HEV sequences within the genus *Orthohepevirus*. The GenBank accession numbers, strains and hosts of the *Orthohepevirus C* HEV genotype C1 strains are indicated. The scale bar indicates phylogenetic distances in nucleotide substitutions per site. Bootstrap values equal and over 70% are indicated. An asterisk marks rat HEV sequences described in this study. Geographical origins of samples are also indicated based on Fig. 1.

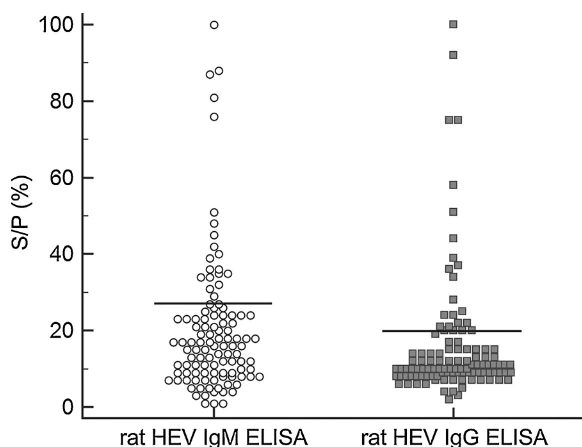

Fig. 3. Results of serological investigation of blood samples of wild rats trapped in Lithuania (n = 109). Cut-off values are indicated by horizontal lines (IgM ELISA: 27%; IgG ELISA: 20%).

(Johne et al., 2012). The observed high seroprevalence suggest a high rate of rat HEV circulation in the examined rat population. Thus, the population of Lithuanian wild rats may represent a promising object for future studies of rat HEV transmission routes. The comparison of results of serological and molecular investigation did not show an association between the presence of rat HEV RNA and rat HEV-specific antibodies. Only 4.6% of rats examined were positive for both rat HEV RNA and anti-rat HEV antibodies, while 26.6% and 3.7% of rats were only anti-rat HEV antibody positive and only rat HEV RNA positive, respectively. These results are in accordance with previous field and experimental studies that rat HEV infection in Norway rats are most likely non-persistent (Purcell et al., 2011; Johne et al., 2012). In contrast to Norway rats there are still very limited data on the association of anti-HEV antibody and RNA detection in Black rats in Europe (Ryll et al., 2017). In our study we observed a similar pattern of rat HEV infection markers in Black rats as that previously reported in Norway rats (Johne et al., 2012). This observation implies that the non-persistent rat HEV infection in individual rats is independent of rat species.

5. Conclusion

The current study demonstrated the presence of rat HEV infection in Black rats and Norway rats from Lithuania. Phylogenetic analysis of the newly identified rat HEV sequences showed a highly related clustering with genomic sequences of rat HEV sequences from Germany. Serological analysis using yeast-expressed rat HEV capsid protein revealed high prevalence of rat HEV-specific antibodies in the rat

Table 2
Results of molecular and serological screening of liver and blood samples of wild rats trapped in Lithuania (n = 109).

Region[1] (district, city or village)	Number of samples tested	RT-qPCR positive liver samples	%	Anti-rat HEV IgM positive blood samples	%	Anti-rat HEV IgM and IgG positive blood samples	%	Anti-rat HEV IgG positive blood samples	%
Joniškis dist., Šarūnai	3	0		0		0		0	
Rokiškis dist., Koveliai	2	1		0		0		0	
Rokiškis dist., Laibgaliai	10	0		3		1		1	
Šilutė dist., Grabupiai	6	1		0		0		5	
Utena dist., Vilučiai	1	0		0		0		0	
Vilnius dist.	6	1		0		2		1	
Zarasai dist., Antazavė	13	2		2		1		2	
Zarasai dist., Dusetos	2	0		1		0		1	
Zarasai dist., Pakniškiai	66	4		7		4		3	
Total	109	9	8.3	13	11.9	8	7.3	13	11.9

[1]For details see Fig. 1.

population analyzed. The current investigation increases the knowledge on rat HEV geographical distribution in Europe to the eastern part.

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Author contributions

I.K.-K. and M.S. conceived and designed the experiments, analyzed the data and drafted the paper; A.V., I.K.-K., K.J., M.J., M.S. and P.L.T. performed the research; A.Z. and R.G.U. contributed materials/analysis tools, revised critically the manuscript and approved the version to be submitted.

Conflicts of interest

The authors declare that they have no conflict of interest.

Animal welfare

All applicable institutional, national, and international guidelines for the care and use of experimental animals were followed. The Wistar rats were used under the license no. LT 59–902, permission no. G2-38 obtained from the State Food and Veterinary Service, Vilnius, Lithuania.

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