Research paper

Genome characterization, prevalence, and tissue distribution of astrovirus, hepevirus and norovirus among wild and laboratory rats (*Rattus norvegicus*) and mice in Hungary.

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Highlights ***(Max. 5 bullet points)***

- Viral metagenomics study of faeces from wild rats (*Rattus norvegicus*) in Hungary
- Three RNA viruses (astro-, noro- and hepevirus) were identified and characterized
- The first evidence of these viruses in rat tissues
- The first quantitative and comparative analysis of astro-, noro- and hepeviruses viral loads
- Identification of rat astro- and norovirus in faeces of laboratory rats and mice

Abstract ***(It should not be more than 300 words.)***

Rodents including rats are reservoir of pathogens capable of affecting human health. In this study, faecal and different organ specimens from free-living Norway rats (*Rattus norvegicus*) (N=18) and faecal samples from laboratory rodents (rats N=21 and mice N=20) collected from different geographic areas in Hungary between 2017 and 2020 were investigated by viral metagenomics and conventional PCR methods. The complete genome of three different RNA viruses, rat astrovirus (MW149253), rat norovirus (MW174170) and rat hepevirus (MW149254) were characterized and analysed in details. Rat norovirus was detected in faecal (17.6%) and kidney (7.1%) samples; rat astrovirus in faecal (23.5%) and spleen (13.3%) samples, and rat hepevirus in 43 to 67% of faecal, liver, kidney, lung, heart, muscle, brain and blood samples from Norway rats, respectively. Rat norovirus was also identifiable in 5% of laboratory rats and rat astrovirus in 40% of faecal samples from laboratory mice. Co-infections were found in 28% of wild Norway rats. The highest RNA viral load of astrovirus ($1.81x10^8$ copy/g) and norovirus ($3.49x10^7$ copy/g) were measured in faecal samples; while the highest RNA viral load of hepevirus ($1.16x10^9$ copy/g) was found in liver samples of Norway rats. This study confirms the wide geographic distribution and high prevalence of astrovirus, norovirus and hepevirus among wild rats in Hungary with confirmation of different organ involvement as well as the detection of norovirus and astrovirus in laboratory rats and mice, respectively.

Keywords ***(max. 6 keywords)*****:** rodents, Norway rat, mice, astrovirus, hepevirus, norovirus

1. Introduction

Rodents (order: Rodentia) have been a frequent source of zoonotic infectious diseases in human, due to the fact that approximately 40% of mammalian species are rodents (with ≈2000 living species); that they are found at high prevalence on almost every continent except Antarctica and a few oceanic islands; that they have relatively high reproductive rate (mainly in family Muridae, with 2.7-8.5 young per 19-44 gestation days, and that these animals are frequently in close contact with humans [Meerburg et al., 2009; https://animaldiversity.org/accounts/Rodentia/; Yom-Tov 1985; Dennis and Mead 2009; Gravinatti et al., 2020]. The family Muridae is the most diverse with ~700 species including mice, rats, and gerbils [https://animaldiversity.org/accounts/Muridae/]. The brown rat (*Rattus norvegicus*) is the dominant species in Europe and much of North America with a size up to 28 cm and 300-1000g in weight [https://animaldiversity.org/accounts/Rattus_norvegicus/]. These excellent foragers are omnivores and live in close contact with humans and hence can be common reservoirs of pathogens for human [McFarlane et al., 2012; Chagas et al., 2017; Gravinatti et al., 2020]. Rats combine the adaptations needed for effective urbanisation and the traits that make them efficient reservoirs for a wide range of zoonotic pathogens (Strand and Lundkvist, 2019). As a recent study pointed out, those animals that live closer to humans tend to carry more pathogens (Gibb et al., 2020).

To date several potential human pathogens have been described in rodent including rats [Heyman et al., 2004; Meerburg et. al., 2009; Tonetti et al., 2013; Firth et. al., 2014; Chagas et al., 2017; Clement et al., 2019; Moreira et al., 2019], and this list can be expanded based on the results of viral metagenomics based viral identification and discovery [Sachsenröder et al., 2014; Hansen et al., 2016; Boros et al., 2019]. However, despite advanced molecular techniques, we only know a small fraction of the viral diversity around us making us vulnerable to zoonotic outbreaks (Daszak et al., 2020). For this reason, it is necessary to extend our knowledge about new potential pathogenic viruses, especially in urban environments.

Astroviruses (family *Astroviridae*), caliciviruses (family *Caliciviridae*) and hepeviruses (family *Hepeviridae*) are non-enveloped viruses with monopartite, linear, positive sense, single-

stranded RNA genome, which could be transmitted by the faecal-oral route [https://viralzone.expasy.org]. Astroviruses (AstVs) are classified into two major groups of viruses, genera *Mamastrovirus* and *Avastrovirus* [Guix et al., 2013; Bosch et al., 2014], caliciviruses (CaVs) are subdivided into eleven genera *Bavovirus, Lagovirus*, *Minovirus*, *Nacovirus*, *Nebovirus*, *Norovirus*, *Recovirus*, *Salovirus*, *Sapovirus*, *Valovirus* and *Vesivirus* [Vinjé et al., 2019], while hepeviruses are currently divided into two genera *Orthohepevirus* and *Piscihepevirus* [Smith et al., 2014]. The AstVs, CaVs and hepeviruses have approximately the same genome length (≈6.6-8.5kb) and a poly(A)-tail at their 3'ends, but AstVs and CaVs RNA are flanked at their 5' end by a covalently linked viral protein (VPg), while hepeviruses are flanked by a cap (m7G) structure at the 5'ends [https://viralzone.expasy.org/; Al-Mutairy et al., 2005;Herbert et al., 1997; Kabrane-Lazizi et al., 1999]. The genomes of these viruses contain multiple open reading frames (ORFs) and encode the non-structural proteins (ORF1ab in AstV; ORF1/ORF4 in CaVs and ORF1/ORF3 in hepeviruses) and encode the protein(s) generating the viral capsid (ORF2 in AstV and hepeviruses; ORF2/ORF3 in CaVs) [Graff et al., 2005, 2006; Arias and DuBois 2017; Dryden et al., 2012]. The viral genomes of members of all three families contain well-known structural elements (e.g.: internal-stem loops, stem-loop-II-motif) [Monceyron et al., 1997; Cao and Meng 2010], conserved nucleotide sequences (e.g.: frameshifting and subgenomic RNA (sgRNA) promoter signals) [Jiang et al., 1993; Firth and Atkins 2010; Ding et al., 2018] and amino acid motifs (e.g.: RNA-dependent RNA polymerase conserved sites) [Koonin EV., 1991]. Also common to all three virus families is that, the capsid proteins are translated from a sgRNA molecule using a highly conservative sgRNA translation initiation signal [Monroe et al., 1993;] in astro- and caliciviruses or a highly conserved RNA motif in the *cis*-reactive (CRE) element in hepeviruses [Emmerson et al., 2001, 2013]. The structure and homology of the translated viral capsid protein also show similarity

between astro- and hepeviruses but differ in noroviruses [Koonin et al., 2008; Dong et al., 2011; Kelly et al., 2016].

~~Overall, the members of the aforementioned virus families (AstVs, NoVs and hepeviruses) show homology in their genome organization and phylogenetic position, their replication strategy even more host ranges, too, while their evolutionary origin, development and formation may have occurred in different ways and over time (Koonin et al., 2008; Kelly et al., 2016; Shi et al., 2016, 2018; Dolja et al., 2018).~~

This study reports the complete RNA genome characterization of rat astrovirus (*Astroviridae*), rat hepevirus (*Hepeviridae*) and rat norovirus (*Caliciviridae*), focuses on their phylogenetic analysis and provides molecular epidemiological data about these viruses in faecal and tissue specimens from wild (Norway) rat (*Rattus norvegicus*) collected from urban and rural areas in Hungary as well as in laboratory rats and mice.

2. Materials and Methods

Between July 2017 and January 2020 a total of 18 free-living Norway rats (*Rattus norvegicus*) were trapped in backyard of small livestock farms from five dispersed geographical locations in Hungary [Boros et al., 2019; in this study]. Faecal and different tissue (liver, spleen, kidney, lung, heart, brain, blood and muscle) samples were collected by certified biologist (Sz. S.) from perished animals. Four randomly selected faecal samples from free-living Norway rats (**rat01**, **rat04**, **rat08**, **rat09**) caught in different locations in Hungary were selected for viral metagenomics analysis as a single (pooled) sample [Boros et al., 2019]. 21 faecal samples were also gathered from laboratory rats ("Wistar-type" and "hooded-type") and 20 faecal samples from specific pathogen-free (SPF) laboratory mice, respectively, between May and September 2019, at the University of Pécs, Pécs, Hungary (Boros et al., 2019). Among the SPF mice, there were 5 $Rag1^{tm1Mom}$ (recombination activation gene 1) knock-out, 5 Nude (lack of $Foxn1^{nu}$)

athymic mice, 5 Bc (standard), and 5 c57BL/6J (inbred) laboratory mice held in isolated laboratory environment.

The faecal samples were homogenized mechanically with 0.1M phosphate buffered saline (35-40 V/v%), while tissue samples (≈50-100mg/sample) were placed in centrifuge tubes containing TRI Reagent (MRC, USA), and under refrigerated conditions, were prepared by ultrasonic sonication (output grade 3 for an average of 25 seconds) (Sonicator Cell Distruptor, W-220F, Heat Sytems, New York, USA) for RNA extraction. 200µl supernatant of faecal suspension was filtered through a 0.45µm filter (Millipore), threated with DNases (Turbo DNase, Ambion; Baseline-ZERO, Epicentre; Benzonase, Novagen) and RNase (Fermentas) to digest unprotected (viral-particle protected free) nucleic acids. cDNA library was constructed by ScriptSeqTM v2 RNA-Seq Library Preparation Kit (Epicentre) followed by random PCR amplification and then sequenced using MiSeq Illumina platform [Phan et al., 2013, Boros et al., 2019]. More than 100-bp-long sequence reads (singletons) and assembled contigs were compared to GenBank protein database (BLASTx). Reads with better match than 10^{-5} (E-values) cut-off threshold were categorized based on their best hits.

Total RNA was also extracted using TRI Reagent according the manufacturer's protocol. The final RNA concentration was measured by NanoDrop 2000 spectrophotometer (ThermoFischer, Waltman, MA, USA). The reverse transcription (RT) and polymerase chain reaction (PCR) conditions were described previously [Boros et al., 2011, 2019]. Sequence specific primer-pairs were designed based on the part of read/contig sequence to identify the target RNA virus in the pool, then different nucleic acid amplification techniques were applied to determine the complete genome [Sverdlov and Azhikina, 2005; Liu and Chen, 2007, Boros et al., 2011]. Based on multiple sequence alignments of reference and study sequence strains degenerate "screening" primer-pairs were designed to explore study strains or homologues in the available samples (Table S1). PCR-products were directly sequenced with BigDye

Terminator v1.1 (ThermoFisher) and run on an automated Sanger sequencer (Genetic Analyzer 3500, Applied Biosystems, Hitachi, Japan).

Viral RNA concentration was determined by qPCR (Rotor-Gene Q MDx, 5plex, Qiagen, Hilden, Germany) using Maxima SYBR Green qPCR Master Mix (ThermoFisher Scientific, USA). Reference standards were created and measured by NanoDrop One (ThermoFisher Scientific, USA) in three technical repeats (Table S1) (Boros et al., 2019). Data were analysed by Rotor-Gene Q Software 2.3.1.49. and the RNA concentration (copy/µg) were calculated from Ct-values.

Representative and complete astro-, noro- and hepevirus strains were collected from GenBank database open reading frames (ORFs) were predicted by the NCBI ORF finder with default settings (https://www.ncbi.nlm.nih.gov/orffinder). The amino acid (aa) sequences were aligned with Multiple Alignment using Fast Fourier Transform application of EMBL-EBI (https://www.ebi.ac.uk/Tools/msa/mafft/) using default settings and the alignments were used for phylogenetic and evolutionary analysis. The aa aligments were pre-tested with IQ-TREE software, Model selection tab using Bayesian (BIC) criteria (http://iqtree.cibiv.univie.ac.at) [Trifinopoulos et al., 2016]. Dendrograms were conducted by MEGA X with Maximum-likelihood method and the IQ-TREE optimized model set-up (see under phylogenetic tree descriptions) [Kumar, Stecher, Li, Knyaz, and Tamura 2018]. Bootstrap (UltraFast) values were determined for 1000 replicates using the whole data subset and the tree was drawn to scale, with branch length measured in the number of substitutions per site with the help of IQ-TREE server. The closest relative search of the study strain was done using NCBI Blast(p) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide (nt) or aa sequence comparisons and identity calculations were conducted by Needleman-Wunsch Global Alignment method (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the similarity score (%) were calculated with SimPlot (ver) [Lole et al., 1999]. The aa sequence analysis was carried out using profile hidden

Markov Model (HMMER) [https://www.ebi.ac.uk/Tools/hmmer/; Potter et al., 2018]. The prediction of transmembrane domains was analysed by Protter web services [http://wlab.ethz.ch/protter/start/, Omasits et al., 2014] and the nuclear localization signals searched by NLStradamus web service (http://www.moseslab.csb.utoronto.ca/NLStradamus/; Nguyen et al., 2009]. The protein folding prediction was carried out using FoldIndex [https://fold.weizmann.ac.il/fldbin/findex; Prilusky et al., 2005], PONDR (predictor of Natural Disordered Regions, http://www.pondr.com/) and the promising novel IUPrad2A webserver [https://iupred2a.elte.hu/, Erdős and Dosztányi 2020]. GeneDoc and CorelDraw X6 were used to edit sequences and annotate phylogenetic trees/graphs [Nicolas and Nicolas, 1997].

The complete genome sequence of the rat astrovirus strain NR/rat01-AstV/HUN/2017 (MW149253), rat norovirus strain NR/rat01-NoV/HUN/2017 (MW174170) and rat hepevirus strain NR/rat08-HEV/HUN/2018 (MW149254) have been deposited into GenBank.

3. Results

Using sequence similarity a total of 19,181 viral reads were derived from nucleic acids randomly amplified from viral particles from the feces of four free-living Norway rat (**rat01**, **rat04**, **rat08** and **rat09**) (Suppl.-Fig.1A). 68.4% and 11.6% of the total reads could be identified as originating from DNA and RNA viral families, respectively, while the source of 20% of the read could not be identified. Among the RNA containing virus families only *Astroviridae, Caliciviridae, Hepeviridae, Nodaviridiae* and *Picornaviridae* are known to infect vertebrates. The picornavirus-related (cardiovirus, kobuvirus and rosavirus) reads have been previously described (Boros et al., 2019). Here we present an analysis of astrovirus-, hepevirus- and calicivirus-related viruses (Suppl.-Fig.1B-D) found in the same specimens.

3.1. Astrovirus in free-living Norway rats

A total of 29 astrovirus-related sequence reads could be aligned to rodent astrovirus strain HK-1797F (KT946732)(Suppl.-Fig.1B). The resulting complete RNA genome of

Norway rat astrovirus strain NR/rat01-AstV/HUN/2017 (MW149253) is 6251-nt-long encoding three open reading frames (ORFs) whereas ORF1a/ORF1b/ORF2 is 2226nt (741aa), 1419nt (472aa) and 2445nt (814aa) in lengths, respectively. (Fig.1A). The RNA genome is composed of A (24%), U (26.1%), G (28%) and C (21.9%) with a GC content of 49.9%.

The 5'UTR is 44-nt-long and the expected conserved 5'-CCAAAG was identified [Finkbeiner et al., 2008]. The putative in-frame AUG initiation codon of ORF1a was found in an optimal Kozak context GAAG**A_{45}UG**UAC (start codon in bold). The most significant match of ORF1a is found to be the NS1A_HASV1 target in human astrovirus 1 (NC_001943) with E-value 3.1e-79 using with PHMMER search. In ORF1a, the coiled-coil structure (CC) is identifiable at aa position 104-131 and a total of five transmembrane domains (TM) present in aa positions at Trp_{147}-Ala_{166}, Tyr_{204}-Tyr_{221}, Thr_{227}-Leu_{247}, Ile_{259}-Val_{283}, Phe_{303}-Leu_{332}. ORF1a showed the closest match to trypsin-like peptidase domain (PF13365.6) [Potter et al., 2018]. Compared to the trypsin-like protease (TLP) of human astrovirus 1, both the TLP protein at aa position 401-521 and the conserved catalytic triad [His(H)-Asp(D)-Ser(S)] aa residues H_{415}, D_{444} and S_{507} [Kiang and Matsui, 2002] were identified in strain NR/rat01-AstV/HUN/2017. Two potential nuclear localization signals (NLS) at aa positions K_{613}-K_{643} and the intrinsically disordered viral protein genome-linked (VPg) at aa position 608-741 with the conserved T_{646}EQEY motif are detected in ORF1a (Fig.1A, Suppl.-Fig.2A). The conserved aa motifs of oligomerization, polymerase interacting domains and the potential hypervariable region (HVR) could not be identified because of very low sequence identity [Guix et al., 2008; Fuentes et al., 2011]. The conserved heptamer +1/-1 frameshifting signal (FS: A_{2219}AAAAAC) was observed in rat astrovirus (Fig.1A) [Jiang et al., 1993].

The ORF1b is in different frame and have no overlapping regions with ORF1a. The putative initiation codon of ORF1b is found in CCG**A_{2351}UG**AAU (start codon in bold). The most significant match of ORF1b is NS1AB_HASV1 of astrovirus 1 with E-value 1.8e-190

using with PHMMER search. In ORF1b, the RNA-dependent RNA polymerase (RdRp) was detected between aa position 79-360 with its active site D_{329}, while further conserved aa motifs: D_{223}WTRFD, double P_{274}SG-P_{286}SG and Y_{327}GDD are also present (Fig.1) [Koonin, 1991; Méndez et al., 2013].

The subgenomic (SG) promoter nt sequence U_{3732}AUGGUGGGGUGGACCAAAA was identifiable upstream in the rat astrovirus capsid proteins encoding ORF2, while downstream the ORF2 initiation start codon starts with GUG**A$_{3759}$UG**GCA (start codon in bold) [Jonassen et al., 2001; Kapoor et al., 2009]. The ORF1b/ORF2 is partially overlaps and the most significant match was to CAPSD_HASV1 of human astrovirus 1 with E-value 1.7e-74 using with PHMMER search and showing the characteristics of astrovirus capsid protein. A total of seven potential Caspase (cysteine-aspartic acid proteases) cleavage sites (threshold score >0.5) were detected by Cascleave (https://sunflower.kuicr.kyoto-u.ac.jp/~sjn/Cascleave/index.html) at ATV**D$_{282}$**AKAG, MIV**D$_{327}$**SAVS, NFE**D$_{378}$**AQNN, PAP**D$_{430}$**GPLL, EVA**D$_{453}$**AVNF, YLV**D$_{772}$**GCSP and RMT**D$_{799}$**GSGA, and a total of 68 potential trypsin cleavage sites were identified (data not shown) [Méndez et al., 2004; Arias and DuBois 2017]. The conserved s2m (stem-loop II) genetic element [Tengs et al., 2013] was not present in the 3'UTR of rat astrovirus strain NR/rat01-AstV/HUN/2017.

The overall percent identity of strain NR/rat01-AstV/HUN/2017 was 78% at nt level to the reference rodent astrovirus sequence (Fig.1A). Based on the putative ORF1a/ORF1b/ORF2 aa sequences of strain NR/rat01-AstV/HUN/2017 showed 82.15%, 91.95% and 71.97% aa identity to the corresponding proteins of representative rodent astrovirus strains. The phylogenetic analysis based on the complete aa sequences of the ORF1a/ORF1b and ORF2 proteins, confirms that the rat astrovirus strain NR/rat01-AstV/HUN/2017 from Hungary can be classified along with rodent astroviruses in genus *Mamastrovirus* (Fig.2A).

3.2. Hepevirus in free-living Norway rats

From the total of 273 hepevirus-related sequence reads only 84-reads could be aligned to rat hepevirus strain LA-B350 (KM516906) (*Orthohepevirus C*) with the best query coverage (Suppl.-Fig.1B). The complete RNA genome of Norway rat hepevirus strain NR/rat08-HEV/HUN/2018 (MW149254) is 6945-nt-long encoding four potential open reading frames (ORFs) whereas ORF1/ORF4 is completely overlap and 4905nt (1634aa) and 513nt (170aa) in lengths and ORF2/ORF3 is also overlap and 1935nt (644aa) and 309nt (102aa) in lengths, respectively (Fig.1B). Checking the 273 hepevirus-related reads, 238 (87.5%) reads could be aligned, while 34 (12.5%) reads could not be aligned to the study strain (data not shown). The RNA genome is composed of A (18.6%), U (23.3%), G (29.4%) and C (28.7%) with a GC content of 58%.

The 5'UTR is 10-nt-long and the putative in-frame initiation site of ORF1 starts with CCG**A_{11}UG**GAG (start codon in bold). The most significant match of ORF1 is to POLN_HEVCH target in genotype 1 HEV (NC_001434) with E-value 0 using with PHMMER search. In the term of function, the viral methyltransferase (PF01660.17), the cysteine protease (PF05417.11), the Macro domain (PF01661.21), viral (superfamily 1) RNA helicase (PF01443.18) and the RdRp (PF00978.21) domains were detected (Fig.1B). For further in-depth analysis the study strain was aligned with the reference strain Burma-1 (NC_001434), and both the hypervariable (HVR) and the poly-proline (PPR) regions were also identifiable in rat hepevirus strain NR/rat08-HEV/HUN/2018. Comparing the corresponded domains/regions of the study strain with the Burma-1 strain several conserved aa motifs (motif_I-VI) could be observed (Fig.1B). The conserved site of the cis-acting replication element (CRE) and the transcription start site (TSS) is detected in nt position at 4910 (G_{4910}AAUAACAC) and at 4934 (UG$_{4934}$U), respectively [Ding et al., 2018]. Downstream the genome two overlapping regions, the viral capsid protein (ORF2) and the viroporin (ORF3) were encoded and translated from different reading frame in the study strain [Ding et al., 2018]. The putative AUG initiation sites

of ORF2 and ORF3 starts with GGA**A**$_{4943}$**UG**CGU and AGC**A**$_{4932}$**UG**UGU (start codons in bold). The most significant match of ORF2 is to CAPSD_HEVCH of HEV genotype 1 (M94177) with E-value 8.2e-225 using PHMMER search. In the term of function, only the 5'conserved part (S-domain) could be identified by the automatic search; however, the conserved ADTLLGLPTDLVSNA (motif_VII) in the P1 subdomain and the potential B-cell type epitopes VTIPHD and FDNQH in P2 subdomain and additional three N-glycosylation sites (NLS and NLT in S-domain and NTT in P1 subdomain) could be detected by in-depth comparative analysis (Fig.1B) [Guu et al., 2009; He et al., 2008; Wang et al., 2017]. Analysing the putative ORF3 and ORF4 proteins using PHMMER, no significant match was found to hepeviruses. Only the comparative analysis with the corresponding regions of the reference Burma 1 (NC_001434) and other rat hepeviruses gave us some information about both proteins. Using this method, the conserved CVSLSCSCFCCSCRCCSRP and the PSYPMP aa motifs in the ORF3 protein and the LLS, GRIRYSALFIIFWRR, LLM and LLI conserved aa motifs in the ORF4 protein were found in the study strain (Fig.1B) [Mulyanto et al., 2014; Takahashi et al., 2016; Tanggis et al., 2018]. The overall percent identity of strain NR/rat08-HEV/HUN/2018 was 85.2% at nt level to the most closely related rat hepevirus strain LA-B350 (KM516906) sequence (Fig.1B). Based on the putative aa sequence of ORF1/ORF2/ORF3/ORF4 of strain NR/rat08-HEV/HUN/2018 it showed 94.6%, 97.7%, 93.1% and 88.7% aa identity to the corresponding proteins of rat hepevirus strains ratESOLO-006SF (AB847308), R68 (GU345043), rat/Mu09/0434/DEU/2010 (JN167538) and GZ95 (MH729810), respectively. The phylogenetic analysis of the ORF1/ORF2 derived complete aa sequences revealed that the rat hepevirus strain NR/rat08-HEV/HUN/2018 from Hungary is a member of the HEV-C1 in species *Orthohepevirus C* (Fig.2B).

3.3. Norovirus in free-living Norway rats

From the total of 138 norovirus-related sequence reads 54 could be aligned to rat norovirus strain Rn/GV/HKU_KT/HKG/2012 (JX486102) with the best query coverage (Suppl.-Fig.1B). The complete RNA genome of Norway rat norovirus strain NR/rat01-NoV/HUN/2017 (MW174170) is 7567-nt-long encoding four open reading frames (ORFs) whereas ORF1$^{frame+2}$/ORF2$^{frame+3}$/ORF3$^{frame+2}$ and ORF4$^{frame+1}$ is 5106nt (1701aa), 1767nt (588aa), 657nt (218aa) and 669nt (222aa) in lengths, respectively (Fig.1C). Back-checking the 138 norovirus-related reads, 122 (88.4%) could be aligned, while 16 (11.5%) reads could not be aligned to the study strain (data not shown). The RNA genome is composed of A (23%), U (22.9%), G (26.8%) and C (27.3%) with a GC content of 54.1%.

The 5'UTR is 10-nt-long and the putative initiation site of ORF1 starts with AGG**A$_{11}$UG**GCU (start codon in bold). Analysis of the ORF1 protein yielded a significant match to POLG_NVN68 of Norwalk virus (NC_001959) E-value 0 using with PHMMER search. In the term of function, the region of N-terminal viral polyprotein (PF08405.11) (NS1-2), the RNA helicase (PF00910.22) (NS3), the C37 peptidase (PF05416.12) (NS3) and the RdRp (PF00680.20) (NS7) domains were clearly recognizable (Fig.1C). For more in-depth analysis the ORF1 protein of the study norovirus strain and the corresponded aa sequence of reference type species polyprotein (NP_056820) further norovirus non-structural domains were detected using with Needleman-Wunsch method [Campillay-Véliz et al., 2020]. The p48/N-terminal peptide is the characteristic protein of genus *Norovirus*, which is also identifiable in the rat norovirus sequence. Based on our analysis, the N-terminal disordered proline rich region (Suppl.-Fig.2B) [Lateef et al., 2017], the transmembrane hydrophobic domain (WSFA[...]IFW) [Ettayebi et al., 2003], the H-box (HYSI), NC aa sequence motifs [Hardy ME 2005] and coiled-coil structure (CC 451-474) were identifiable in the NS1-2 peptide (Fig.1C). Several caspase enzyme cleavage and phosphorylation sites were predicted (Fig.1C) [Sosnovtsev et al., 2006]. Among of the potential caspase cleavage sites DHWD*TYKG (probability: 1.027) was the

most probable site based on the analysis and the alignment with the reference norovirus GI polyprotein (NP_056820). The N-terminal part (1-53/70aa) of the NS1-2 protein was disordered (Suppl.-Fig.2B). Analysing the NS1-2 protein with Disorder-Enhanced Phosphorylation Sites Predictor, 22.2% of the threonine (Thr) and 45.5% of the tyrosine (Tyr) were predicted to be phosphorylated [Lateef et al., 2017], but only one tyrosine (Tyr^{51}) residue with high (≈0.95% Depp Score) probability was identified inside the disordered region. Analysing the NTPase protein, the hydrophobic and acidic consensus regions, the NTP-binding motif (motif_I) and additionally the classical motifs of SF3 helicase (motif_II-IV) were identified in the study strain (Fig.1C) [Pfister and Wimmer 2001]. Furthermore, three other motifs (motifs_V-VII) preceding the classical motifs were highly conserved and identifiable in strain NR/rat01-NoV/HUN/2017 (Fig.1C) [Pfister and Wimmer 2001]. The p22 (p20) (NS4) protein was predicted as non-myristoylated and no other conserved motif was detected [Belliot et al., 2003]. The viral protein genome-linked (VPg, NS5) and the proteinase ($3CL^{pro}$) (NS6) proteins were clearly mapped in the study strain with its TDEE**Y** (RNA linkage in bold) and the GDCG, respectively, and the latter contains an active nucleophilic residue [Burroughs and Brown 1978; Liu et al., 1996; Someya et al., 2002; Godfellow I., 2011]. A putative new conserved motif (PAYLG) was also identified in rat norovirus [Xie et al., 2014]. Finally, the active site [Ng et al., 2004] and several conserved aa motifs of RdRp (NS7) of caliciviruses were identified in rat norovirus as well (Fig.1C) [Argos et al., 1984; Koonin EV., 1991; Fukushi et al., 2004]. The major viral capsid protein (VP1 in ORF2) and the minor structural protein (VP2 in ORF3) coding regions start with GUU$\mathbf{A_{5103}UG}$AGG and AUC$\mathbf{A_{6746}UG}$AGU (start codon in bold), respectively. Analysis of the ORF2 and ORF3 proteins yielded a significant match to CAPSD_NVN68 and to VP2_NVN68 of Norwalk virus (M87661) E-values 2.2e-82 and 5e-13 using PHMMER search. In the term of function, the VP1 viral capsid protein encoded ORF2 and the VP2 viral capsid protein encoded ORF3 significantly matched to Calici_coat

(PF00915.20) and the RNA_capsid (PF03035.14). The rat norovirus presumably encodes a viral factor 1 protein (VF1 in ORF4). The potential gene starts with GUG**A$_{5116}$UG**CUG (start codon in bold), but significant match was not detected by PHMMER search. The overall percent identity of strain NR/rat01-NoV/HUN/2017 was 89% at nt level to the closest relative rat norovirus strain RN/GV/HKU_KT/HKG/2012 (JX486102) sequence (Fig.1C). Based on the putative aa sequence of ORF1/ORF2/ORF3 of the study strain NR/rat01-NoV/HUN/2017 showed 97.7%, 88.3% and 96.8% aa identity to the corresponding proteins of rat norovirus strain RN/GV/HKU_KT/HKG/2012 (JX486102) and Rn/GV/HKU_CT2/HKG/2011 (JX486101), respectively, but the ORF4 showed only 54.3% aa identity to the ORF4 protein of murine norovirus strain Mu/NoV/GV/MNV1/2002/USA (DQ285629). The phylogenetic analysis of the ORF1/ORF2/ORF3 derived complete aa sequences using the corresponding proteins of representative members of GI-GVI noroviruses, rat norovirus strain NR/rat01-NoV/HUN/2017 (MW174170) from Hungary is assigned to norovirus genotype GV in genus *Norovirus* (Fig.2C).

3.4. Molecular epidemiology of astro-, noro-, and hepeviruses from faecal and different organ samples collected from wild and laboratory animals

Free-living wild rats were trapped from geographically diverse areas of Hungary, mainly (14/18) from a small town, Hajdúböszörmény, between 2017 and 2020 (Fig.3). These animals were captured by dog/poisoning/trap in suburb region four houses apart (one living space for that animals). Based on the study strains real-time PCR primer-pairs and screening primer-pairs involving close relative sequences were designed for quantitative RNA determination and molecular epidemiological study (Suppl.-Table1). Using the screening primer pairs, 23.5% and 13.3% of the faecal and spleen samples contained rat astrovirus RNA; 17.6% and 7% of the faecal and kidney samples, respectively, were positive for rat norovirus RNA, while rat hepevirus RNA could be detected in 53% of faecal, 50% of liver, 66.6% of

spleen, 42.8% of kidney, 46.2% of lung, 50% of heart, 37.5% of muscle, 50% of brain and 50% of blood samples (Fig.3). Co-infections were detected in five rats. Rat astrovirus and norovirus were found in **rat01** and **rat05**, rat astrovirus and hepevirus were identified in **rat10** and **HB5**, while rat norovirus and hepevirus were identifiable in **HB6** animals (Fig.3). Rat astrovirus and norovirus was found mostly in the faecal samples of the animals, but rat astrovirus could also be detected from spleen of **rat01** and **rat10**, while rat norovirus was found in kidney of **rat01**.

The screening of the laboratory rats and SPF mice faecal samples showed interesting results. While rat hepevirus could not be detected from faecal samples of these laboratory animals, rat norovirus was identified in "Wistar-type" rat **WP2** and rat astrovirus was identified in all of the five **Rag1**, one **Nude** and two **Bc** SPF mice faecal samples (data not shown). All amplicons were confirmed by nucleotide sequencing and showed more than 95% nt identities to study strains.

To obtain the quantitative viral RNA concentration of the RT-PCR-positive samples, specific (semi)nested primer pairs were designed to the read detection primer pairs (Suppl.-Table1, Suppl.Fig.3). First of all, the mass of the faecal or tissue samples were measured in µg, secondly, the cDNAs were synthetized by specific reverse primers then nested-PCR products were amplified. Based on the Ct values and the mass of the samples the RNA concentration was measured in each case and calculated in viral copy number per µg tissue (Suppl.Fig.3). The virus concentration was determined in each of the astrovirus and norovirus positive samples, while in the case of hepevirus the viral RNA could not be quantified from 1 faecal (**rat03**), 5 liver (**rat07**, **HB1**, **HB4**, **HB5**, **HB7**), 2 spleen (**HB5**, **HB6**), 1 kidney (**HB3**), 1 heart (**HB4**) samples. Briefly, astrovirus was measured from faecal samples and spleen in median $1.65x10^6$ (min.: $5.93x10^4$, max.: $1.81x10^8$) and $1.38x10^7$ (min.: $1.05x10^3$, max.: $2.75x10^7$) copies per ug, while norovirus was detected in faecal samples and kidney in median $5.55x10^5$ (min.: $6.62x10^2$, max.: $3.49x10^7$) and $1.08x10^3$ copies per gram, respectively. Rat hepevirus RNA per µg tissue

sample could be detected from faecal (median: $7.58x10^5$, min.: $2.85x10^3$, max.: $1.49x10^8$), from liver (median: $1.83x10^5$, min.: $1.9x10^4$, max.: $1.16x10^9$), from spleen (median: $1.88x10^4$, min.: 1.29x10, max.: $2.12x10^6$), from kidney (median: $2.3x10^3$, min.: 6.15, max.: $1.98x10^5$), from lung (median: $1.92x10^4$, min.: 2.23x10, max.: $5.32x10^4$), from heart (median: $2.49x10^3$, min.: $4.81x10^2$, max.: $5.76x10^6$), from muscle (median: $1.92x10^2$, min.: $1.36x10^2$, max.: $5.1x10^6$), from brain (median: $7.42x10^4$, min.: $2.95x10^1$, max.: $6.33x10^6$), from blood (median: $1.45x10^5$, min.: 8.67x10, max.: $2.91x10^5$) samples. At the highest copy number of astrovirus could be detected from faecal sample of **HB5**, of norovirus from faecal sample of **rat01**, while hepevirus could be detected from liver sample of **rat08**. In the samples where co-infection was found, the concentration of the detected viruses was also found to be high. No virus concentration determination was performed in laboratory animals. Details of the measurements and calculations are given in the supplementary file (Suppl.-Fig.3).

4. Discussion

Rats can be reservoirs of a wide range of pathogens, including known human and newly discovered viruses [Meerburg et. al., 2009; McFarlane et al., 2012; Firth et. al., 2014; Sachsenröder et al., 2014; Hansen et al., 2016; Chagas et al., 2017; Boros et al., 2019; Gravinatti et al., 2020]. Among rodents, the role of rats in the transmission and spread of pathogens is unquestionable. *Rattus rattus* and *R. norvegicus* are two of the most commonly found rodent species in human settlements and have long been known as major reservoirs for zoonotic agents [Morse 1995]. However, the microbial "flora" of these synanthropic animals are not fully understood and they potentially carry further agents which could pose a risk to human health. A large scale study has recently shown that rodents living in close vicinity of humans usually harbour more zoonotic pathogens than the naturally living ones (Gibb et al., 2020) .

In this research, three RNA viruses belonging to three different virus families, rat astrovirus strain NR/rat01-AstV/HUN/2017 (MW149253) in family *Astroviridae*, rat norovirus strain NR/rat01-NoV/HUN/2017 (MW174170) in family *Caliciviridae*, and rat hepevirus strain NR/rat08-HEV/HUN/2018 (MW149254) in family *Hepeviridae* were characterized and discussed in details. To date, relatively small number of complete genomes of rat astrovirus (N=15), norovirus (N=2) and hepevirus (N=17), respectively, were reported in GenBank.

Several different astroviruses have been described in wild and laboratory rodents to date and new ones are constantly being discovered [Kjeldsberg and Hem 1985; Chu et al., 2010; Farkas et al., 2012; Yokoyama et al., 2012; Ng et al. 2013; Sachsenröder et al., 2014; Hansen et al., 2016; Boros et al., 2019]. The phylogenetically closest strain of astrovirus sequence to NR/rat01-AstV/HUN/2017 was described in China in 2017 [To et al., 2017]. In that study, four new astrovirus groups could be detected from six different rat species (*Rattus andamanensi*, *Bandicota indica*, *Niviventer fulvescen*, *N. coxingi*, *Rattus rattus* and *R. norvegicus*) from a total of 562 rectal swabs. RoAstV-HK-1893F (KT946733) from "cluster D" [To et al., 2017] collected from *Rattus andamanensi* and *Bandicota indica*, was highly similar to our astrovirus study strain from *R. norvegicus*. At the time of writing the manuscript, additional astroviruses have been described from rats and three of them (MT549859, MT549858 and MT549860 unpublished data) identified from *R. norvegicus* are phylogenetically closely related to our sequence. By analysing the available complete genomes of rat astrovirus sequences, in addition to the "cluster D", the formation of an additional group is conceivable. Interestingly, further, genetically diverse astroviruses have also been detected from *R. norvegicus* [Chu et al., 2010; To et al., 2017 and an unpublished astrovirus sequence (MT549855)], unrelated to our study sequence. This means that several different (geno)types of astroviruses are present and circulate at the same time in wild Norway rats. According to two Chinese studies, the prevalence of

astroviruses in *R. norvegicus* was consistently low (1.6-2.2%) in faeces and seemed that *R. norvegicus* was not susceptible to astrovirus infection [Chu et al., 2010; To et al., 2017]. In contrast, the detection rate of rat astrovirus from faeces in our study was higher (23.5%) and could be much higher if we calculate all known and unknown astrovirus variants. In addition, our study revealed that, astrovirus or viral RNA could pass through the intestine and enter into the bloodstream and causes disseminated infections involving the spleen. Except for our research, no previous study have reported the concentration of astrovirus RNA in rat tissues. The rat astrovirus was postulated to be potentially infectious for human [Chu et al. 2010]. Our finding is of public health interest, since it raises the possibility of long-term astrovirus infection of rats. Testing of more rats collected from different areas should be needed to obtain a more accurate picture of the prevalence, genetic distribution of rat astroviruses and test the possibility of human infections.

Noroviruses have been detected in many mammalian species and are among the most important pathogens in human acute gastroenteritis [Wobus et al., 2004; Sosnovtsev et al., 2006; McFadden et al., 2011; Green 2013; Wu et al., 2016; Chhabra et al., 2019, 2020; ICTV Online: Norovirus]. The first rodent norovirus Mu/NoV/GV/MNV1/2002/USA (NC_008311) was identified from interferon-alpha/beta/gamma receptor knock-out mouse in the USA in 2002 [Karst et al., 2003; Wobus et al., 2004], while the first rat noroviruses (JX486102 and JX486101) were discovered in China in 2011 [Tse et al., 2012]. The rat norovirus strain NR/rat01-NoV/HUN/2017 (MW174170) has the closest genetic identity to Rn/GV/HKU_KT/HKG/2012 (JX486102) norovirus sequence identified from *R. norvegicus* in China in 2011 that represented a novel cluster within the GV genogroup [Tse et al., 2012]. To date, norovirus has been detected in only two rat species *R. rattus* and *R. norvegicus* [Tse et al., 2012; Tsunesumi et al., 2012]. The partial ORF1/ORF2 sequence of strain Rat47 (AB684736) found in the *R. rattus* species have been described in Japan in 2011, while the two full-genome

virus sequences of strain Rn/GV/HKU_CT2/HKG/2011 (JX486101) and strain Rn/GV/HKU_KT/HKG/2012 (JX486102) found in *R. norvegicus* in China. In our study, rat norovirus was detected in 17.6% of the collected faecal samples, and interestingly, norovirus RNA was detectable extra intestinally in the kidney of an animal for the first time. The concentration of norovirus in the faecal samples of the three animals ranged from $6.62x10^2$-$3.49x10^7$ copy/g, whereas the virus concentration was $1.08x10^3$ copy/g in the kidney. No previous study has reported the norovirus concentration in rats. However, the cDNA viral load of GI and GII norovirus genotypes responsible for human infections was measured in a study of faecal viral load and norovirus-associated gastroenteritis, where the median concentration was $8.4x10^5$ cDNA copy for GI genotypes and $3x10^8$ cDNA copy for GII genotypes per gram of faecal specimen [Chan et al., 2006]. Converting our results for comparison, the cDNA load was $6.22x10^6$; $1.18x10^2$ and $9.91x10^4$ per gram faeces of rat01, rat05 and HB5, respectively while the cDNA load was $1.93x10^2$ per gram kidney of rat01. This means that the amount of norovirus excreted in rat faeces is equal to the amount of noroviruses of the GI genotype detected in acute human infections. The level of norovirus concentration measured in the kidney raises the possibility that norovirus may also cause extraintestinal infection. Based on the sequence analysis of the norovirus genome we found the virulence factor 1 (VF1 formerly called ORF4) described previously exclusively for murine noroviruses [McFadden et al., 2011]. This VF1 is also present in the rat norovirus sequences. Based on the current phylogenetics of GV noroviruses, it is divided into two subgroups, murine and rat noroviruses, and currently these groups show host species specificity [Moreira et al., 2019].

Hepeviruses have been detected in a wide range of vertebrates including human causing acute hepatitis and chronic infection in immunocompromised individuals [Khuroo 2011; Schlosser et al., 2011; Primadharsini et al., 2019; ICTV Online: Hepeviridae]. Hepeviruses in species *Orthohepevirus C*, mainly infects rats and ferrets; however, the importance of these

group of viruses increased since the first human infections have been confirmed [Sridhar et al., 2018; Reuter et al., 2020]. NR/rat08-HEV/HUN/2018 (MW149254) has the closest identity to rat hepevirus sequence from Germany in 2009 [Johne et al., 2012]. In their survey, the prevalence of rat hepevirus was found between 2.9 to 35.3% after testing a total of 147 blood and liver tissue samples of *R. norvegicus*. These data are in line with our study where 50-50% prevalence of rat hepevirus was found in blood and liver samples, respectively. Moreover, rat hepevirus have been identified for the first time from spleen, kidney, lung, heart, muscle and brain tissue samples presumably causing disseminated infection through the bloodstream. The amount of virus RNA load determined in rat organs can be considered high in light of the available literature data [Borkakoti et al., 2013]. The viral load of human hepatitis E virus was found to be 12.73 copies per millilitre in a non-pregnant patient's group with acute viral hepatitis [Borkakoti et al., 2013]. In contrast, converting our data to this unit, the median $4.8x10^5$ and $2.23x10^5$ copies per millilitre measured in rat faecal and blood samples were 10,000-fold higher than those measured for hepatitis E virus in human.

This study complements the results of our previous investigation, where the molecular epidemiology and complete genome characterization of three different picornaviruses (cardiovirus, kobuvirus and rosavirus) was reported in the same specimens of free-living wild and laboratory rats [Boros et al., 2019]. According to our results, overall, 5 different RNA viruses (3 picornaviruses, astrovirus and norovirus) from three different virus families could be identified from the faecal sample of the same animal **rat05**, but there were two rats (**rat06** and **rat08**) where 4 different viruses (3 picornaviruses and norovirus or hepevirus) were present in the faeces. We were able to describe at least two pathogens belonging to different virus families in four animals (**rat01**, **rat02**, **rat03**, **rat07**) [in this study, Wolf et al., 2013; Boros et al. 2019]. These results reflect co-circulation and co-infection of different RNA viruses in wild rat

population indicating the probable role of rats as long-term reservoirs of these viruses. [Wolf et al., 2013]

In addition to the study of wild rats, another interesting area is the study of virome of laboratory mice and rats, including gnotobiotic animals, which are widely used and held in laboratories in the world for experimental studies [Baker et al., 2013]. To date, some RNA viruses like hantavirus have been identified in laboratory rats [Clement et al., 2019], astrovirus and norovirus could be detected in laboratory mice [Ng et al., 2013; Hayashimoto et al., 2013; Ingle et al., 2019] and three different picornaviruses (cardiovirus, kobuvirus and rosavirus) were also found in our previous study among the tested laboratory rats [Boros et al., 2019]. While hepevirus was not identifiable in laboratory rodents, norovirus could be detected in experimental rats and rat astrovirus in a high percentage of laboratory mice. Our results further reinforce that norovirus and astrovirus could circulate in experimental rats and mice, respectively, providing important health risk information for those who work with these experimental animals. Based on these, testing of these animals for further pathogens is highly recommended [Livingstone and Riley 2003].

5. Conclusion

In conclusion, our study demonstrates the presence of astrovirus, hepevirus and norovirus among wild (*Rattus norvegicus*) and laboratory rats and mice in Hungary with confirmation of different organ involvement. These urbanised animals live in rural area (farm animal pens) and metropolitan environment (landfills and sewers, streets or even on buses) in close contact with both other animals and humans [Zhou et al., 2014, 2016]. Rodents are specifically ideal hosts for carrying and spreading a wide range of viral pathogens that pose a potential indirect or direct threat to the human population. The more detailed composition of

viruses in these vertebrate hosts may help to understand the evolutionary history and zoonotic potential affecting human health.

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References

Al-Mutairy, B., Walter, J.E., Pothen, A., Mitchell, D.K. (2005). Genome prediction of putative genome-linked viral protein (VPg) of astroviruses. Virus Genes. 31, 21-30. https://doi.org/10.1007/s11262-004-2196-1.

Argos, P., Kamer, G., Nicklin, M.J., Wimmer, E. (1984). Similarity in gene organization and homology between proteins of animal picornaviruses and a plant comovirus suggest common ancestry of these virus families. Nucleic Acids Res. 12, 7251-7267. https://doi.org/10.1093/nar/12.18.7251.

Arias, C.F., DuBois, R.M. (2017). The Astrovirus Capsid: A Review. Viruses. 9, 15. https://doi.org/10.3390/v9010015.

Baker, H.J., Lindsey, J.R., Wesibroth, S.H. (2013). The laboratory rat: Biology and Diseases (Vol.1). Elsevier, Academic Press, eBook ISBN: 9781483268613.

Baldridge, M.T., Nice, T.J., McCune, B.T., Yokoyama, C.C., Kambal, A., Wheadon, M., Diamond, M.S., Ivanova, Y., Artyomov, M., Virgin, H.W. (2015). Commensal microbes and interferon-λ determine persistence of enteric murine norovirus infection. Science. 347, 266-269. https://doi.org/10.1126/science.1258025.

Belliot, G., Sosnovtsev, S.V., Mitra, T., Hammer, C., Garfield, M., Green, K.Y. (2003). In vitro proteolytic processing of the MD145 norovirus ORF1 nonstructural polyprotein yields stable precursors and products similar to those detected in calicivirus-infected cells. J. Virol. 77, 10957-10974. https://doi.org/10.1128/jvi.77.20.10957-10974.2003.

Borkakoti, J., Hazam, R.K., Mohammad, A., Kumar, A., Kar, P. (2012). Does high viral load of hepatitis E virus influence the severity and prognosis of acute liver failure during pregnancy? J. Med. Virol. 85, 620-626. https://doi.org/10.1002/jmv.23508.

Boros, Á., Orlovácz, K., Pankovics, P., Szekeres, S., Földvári, G., Fahsbender, E., Delwart, E., Reuter, G. (2019). Diverse picornaviruses are prevalent among free-living and laboratory rats (Rattus norvegicus) in Hungary and can cause disseminated infections. Infect. Genet. Evol. 75, 103988. https://doi.org/10.1016/j.meegid.2019.103988.

Boros, Á., Pankovics, P., Simmonds, P., Reuter, G. (2011). Novel positive-sense, single-stranded RNA (+ssRNA) virus with di-cistronic genome from intestinal content of freshwater carp (Cyprinus carpio). PLoS One. 6, e29145. https://doi.org/10.1371/journal.pone.0029145.

Bosch, A., Pintó, R.M., Guix, S., (2014). Human astroviruses. Clin. Microbiol. Rev. 27, 1048-1074. https://doi.org/10.1128/CMR.00013-14.

Burroughs, J.N., Brown, F. (1978). Presence of a covalently linked protein on calicivirus RNA. J. Gen. Virol. 41, 443-446. https://doi.org/10.1099/0022-1317-41-2-443.

Campillay-Véliz, C.P., Carvajal, J.J., Avellaneda, A.M., Escobar, D., Covián, C., Kalergis, A.M., Lay, M.K. (2020). Human Norovirus Proteins: Implications in the Replicative Cycle, Pathogenesis, and the Host Immune Response. Front. Immunol. 11, 961. https://doi.org/10.3389/fimmu.2020.00961.

Cao, D., Huang, Y.W., Meng, X.J. (2010). The nucleotides on the stem-loop RNA structure in the junction region of the hepatitis E virus genome are critical for virus replication. J. Virol. 84, :13040-13044. https://doi.org/10.1128/JVI.01475-10.

Chagas, C.R.F., Gonzalez, I.H.L., Favoretto, S.M., Ramos, P.L. (2017). Parasitological surveillance in a rat (Rattus norvegicus) colony in São Paulo Zoo animal house. Ann. Parasitol. 63, 291-297. https://doi.org/10.17420/ap6304.115.

Chan, M.C., Sung, J.J., Lam, R.K., Chan, P.K., Lee, N.L., Lai, R.W., Leung, W.K. (2006). Fecal viral load and norovirus-associated gastroenteritis. Emerg. Infect. Dis. 12, 1278-1280. https://doi.org/10.3201/eid1208.060081.

Chhabra, P., de Graaf, M., Parra, G.I., Chan, M.C., Green, K., Martella, V., Wang, Q., White, P.A., Katayama, K., Vennema, H., Koopmans, M.P.G., Vinjé, J. (2019). Updated classification of norovirus genogroups and genotypes. J. Gen. Virol. 100, 1393-1406. https://doi.org/10.1099/jgv.0.001318.

Chhabra, P., Graaf, M., Parra, G.I., Chan, M.C., Green, K., Martella, V., Wang, Q., White, P.A., Katayama, K., Vennema, H., Koopmans, M.P.G., Vinjé, J. (2020). Corrigendum: Updated classification of norovirus genogroups and genotypes. J. Gen. Virol. 101, 893. https://doi.org/10.1099/jgv.0.001475.

Chu, D.K., Chin, A.W., Smith, G.J., Chan, K.H., Guan, Y., Peiris, J.S., Poon, L.L. (2010). Detection of novel astroviruses in urban brown rats and previously known astroviruses in humans. J. Gen. Virol. 91, 2457-2462. https://doi.org/10.1099/vir.0.022764-0.

Clement, J., LeDuc, J.W., Lloyd, G., Reynes, J.M., McElhinney, L., Van Ranst, M., Lee, H.W. (2019). Wild Rats, Laboratory Rats, Pet Rats: Global Seoul Hantavirus Disease Revisited. Viruses. 11, 652. https://doi.org/10.3390/v11070652.

de Oliveira Tonietti, P., da Hora, A.S., Silva, F.D.F., Ferrari, K.L., Brandão, P.E., Richtzenhain, L.J., Gregori, F. (2013). Simultaneous Detection of Group A Rotavirus in Swine and Rat on a Pig Farm in Brazil. The Scientific World Journal, 648406, https://doi.org/10.1155/2013/648406.

Dennis, D.T., Mead, P.S. (2006). Chapter 42 – Plague. in: Guerrant, R.L., Walker, D.H., Weller, P.F. (Eds.), Tropical Infectious Diseases (Second Edition), Principles, Pathogens & Practice. Churchill Livingstone, pp. 471–481.https://doi.org/10.1016/B978-0-443-06668-9.50047-8.

Ding, Q., Nimgaonkar, I., Archer, N.F., Bram, Y., Heller, B., Schwartz, R.E., Ploss, A. (2018). Identification of the Intragenomic Promoter Controlling Hepatitis E Virus Subgenomic RNA Transcription. mBio. 9, e00769-18. https://doi.org/10.1128/mBio.00769-18.

Dolja, V.V., Koonin, E.V. (2018). Metagenomics reshapes the concepts of RNA virus evolution by revealing extensive horizontal virus transfer. Virus. Res. 244, 36-52. https://doi.org/10.1016/j.virusres.2017.10.020.

Dong, J., Dong, L., Méndez, E., Tao, Y. (2011). Crystal structure of the human astrovirus capsid spike. Proc. Natl. Acad. Sci. USA. 108, 12681-12686. https://doi.org/10.1073/pnas.1104834108.

Dryden, K.A., Tihova, M., Nowotny, N., Matsui. S.M., Mendez. E., Yeager. M. (2012). Immature and mature human astrovirus: structure, conformational changes, and similarities to hepatitis E virus. J. Mol. Biol. 422, 650-658. https://doi.org/10.1016/j.jmb.2012.06.029.

Emerson, S.U., Nguyen, H.T., Torian, U., Mather, K., Firth, A.E. (2013). An essential RNA element resides in a central region of hepatitis E virus ORF2. J. Gen. Virol. 94, 1468-1476. https://doi.org/10.1099/vir.0.051870-0.

Emerson, S.U., Zhang, M., Meng, X.J., Nguyen, H, St Claire, M., Govindarajan, S., Huang, Y.K., Purcell, R.H.(2001). Recombinant hepatitis E virus genomes infectious for primates: importance of capping and discovery of a cis-reactive element. Proc. Natl. Acad. Sci. USA. 98, 15270-15275. https://doi.org/10.1073/pnas.251555098.

Erdős, G., Dosztányi, Z. (2020). Analyzing Protein Disorder with IUPred2A. Curr. Protoc. Bioinformatics. 70, e99. https://doi.org/10.1002/cpbi.99.

Ettayebi, K., Hardy, M.E. (2003). Norwalk virus nonstructural protein p48 forms a complex with the SNARE regulator VAP-A and prevents cell surface expression of vesicular stomatitis virus G protein. J. Virol. 77, 11790-11797. https://doi.org/10.1128/jvi.77.21.11790-11797.2003.

Farkas, T., Fey, B., Keller, G., Martella, V., Egyed, L. (2012). Molecular detection of novel astroviruses in wild and laboratory mice. Virus. Genes. 45, 518-525. https://doi.org/10.1007/s11262-012-0803-0.

Finkbeiner, S.R., Kirkwood, C.D., Wang, D. (2008). Complete genome sequence of a highly divergent astrovirus isolated from a child with acute diarrhea. Virol. J. 5, 117. https://doi.org/10.1186/1743-422X-5-117.

Firth, A.E., Atkins, J.F. (2010). Candidates in Astroviruses, Seadornaviruses, Cytorhabdoviruses and Coronaviruses for +1 frame overlapping genes accessed by leaky scanning. Virol. J. 7, 17. https://doi.org/10.1186/1743-422X-7-17.

Firth, C., Bhat, M., Firth, M.A., Williams, S.H., Frye, M.J., Simmonds, P., Conte, J.M., Ng, J., Garcia, J., Bhuva, N.P., Lee, B., Che, X., Quan, P.L., Lipkin, W.I. (2014). Detection of zoonotic pathogens and characterization of novel viruses carried by commensal Rattus norvegicus in New York City. mBio. 5, e01933-14. https://doi.org/10.1128/mBio.01933-14.

Fuentes, C., Guix, S., Bosch, A., Pintó, R.M. (2011). The C-terminal nsP1a protein of human astrovirus is a phosphoprotein that interacts with the viral polymerase. J. Virol. 85, 4470-4479. https://doi.org/10.1128/JVI.01515-10.

Fukushi. S., Kojima, S., Takai, R., Hoshino, F.B., Oka, T., Takeda, N., Katayama, K., Kageyama, T. (2004). Poly(A)- and primer-independent RNA polymerase of Norovirus. J. Virol. 78, 3889-3896. https://doi.org/10.1128/jvi.78.8.3889-3896.2004.

Goodfellow, I. (2011). The genome-linked protein VPg of vertebrate viruses - a multifaceted protein. Curr. Opin. Virol. 1, 355-362. httpa://doi.org/10.1016/j.coviro.2011.09.003.

Graff, J., Nguyen, H., Yu, C., Elkins, W.R., St Claire, M., Purcell, R.H., Emerson, S.U. (2005). The open reading frame 3 gene of hepatitis E virus contains a cis-reactive element and encodes a protein required for infection of macaques. J. Virol. 79, 6680-6689. https://doi.org/10.1128/JVI.79.11.6680-6689.2005.

Graff, J., Torian, U., Nguyen, H., Emerson, S.U. (2006). A bicistronic subgenomic mRNA encodes both the ORF2 and ORF3 proteins of hepatitis E virus. J. Virol. 80, 5919-5926. https://doi.org/10.1128/JVI.00046-06.

Gravinatti, M.L., Barbosa, C.M., Soares, R.M., Gregori, F. (2020). Synanthropic rodents as virus reservoirs and transmitters. Rev. Soc. Bras. Med. Trop. 53, e20190486. https://doi.org/10.1590/0037-8682-0486-2019.

Green, K. (2013). Calicivirdae; the noroviruses. in: Knipe, D.M., Howley, P.M., Cohen, J.I., Griffin, D.E., Lamb, R.A., Martin, M.A., Racaniello, V.R., Roizman, B. (Eds.), Fields virology, 6th ed, Philadelphia, PA: Lippincott Williams & Wilkins, pp. 583–609.

Guix, S., Bosch, A., Pintó, R.M. (2013). Chapter 6 – Astrovirus taxonomy. in: Schultz-Cherry, S. (Ed.), Astrovirus research: Essential Ideas, Everyday Impacts, Future Directions. Springer Science + Business Media, New York, pp. 97-119. https://doi.org/10.1007/978-1-4614-4735-1. ISBN: 978-1-4614-4734-4.

Guix, S., Caballero, S., Fuentes, C., Bosch, A., Pintó, R.M. (2008). Genetic analysis of the hypervariable region of the human astrovirus nsP1a coding region: design of a new RFLP typing method. J. Med. Virol. 80, 306-315. https://doi.org/10.1002/jmv.21058.

Guu, T.S., Liu, Z., Ye, Q., Mata, D.A., Li, K., Yin, C., Zhang, J., Tao, Y.J. (2009). Structure of the hepatitis E virus-like particle suggests mechanisms for virus assembly and receptor binding. Proc. Natl. Acad. Sci. USA. 106, 12992-12997. https://doi.org/10.1073/pnas.0904848106.

Hansen, T.A., Mollerup, S., Nguyen, N.P., White, N.E., Coghlan, M., Alquezar-Planas, D.E., Joshi, T., Jensen, R.H., Fridholm, H., Kjartansdóttir, K.R., Mourier, T., Warnow, T., Belsham, G.J., Bunce, M., Willerslev, E., Nielsen, L.P., Vinner, L., Hansen, A.J. (2016). High diversity of picornaviruses in rats from different continents revealed by deep sequencing. Emerg. Microbes. Infect. 5, e90. https://doi.org/10.1038/emi.2016.90.

Hardy, M.E. (2005). Norovirus protein structure and function. FEMS Microbiol. Lett. 253, 1-8. https://doi.org/10.1016/j.femsle.2005.08.031.

Hayashimoto, N., Morita, H., Ishida, T., Yasuda, M., Kameda, S., Uchida, R., Tanaka, M., Ozawa, M., Sato, A., Takakura, A., Itoh, T., Kagiyama, N. (2013). Current microbiological status of laboratory mice and rats in experimental facilities in Japan. Exp. Anim. 62, 41-48. https://doi.org/10.1538/expanim.62.41.

He, S., Miao, J., Zheng, Z., Wu, T., Xie, M., Tang, M., Zhang, J., Ng, M.H., Xia, N. (2008). Putative receptor-binding sites of hepatitis E virus. J. Gen. Virol. 89, 245-249. https://doi.org/10.1099/vir.0.83308-0.

Herbert, T.P., Brierley, I., Brown, T.D. (1997). Identification of a protein linked to the genomic and subgenomic mRNAs of feline calicivirus and its role in translation. J. Gen. Virol. 78, 1033-1040. https://doi.org/10.1099/0022-1317-78-5-1033.

Heyman, P., Plyusnina, A., Berny, P., Cochez, C., Artois, M., Zizi, M., Pirnay, J.P., Plyusnin, A. (2004). Seoul hantavirus in Europe: first demonstration of the virus genome in wild Rattus norvegicus captured in France. Eur. J. Clin. Microbiol. Infect. Dis. 23, 711-717. https://doi.org/10.1007/s10096-004-1196-3.

Ingle, H., Lee, S., Ai, T., Orvedahl, A., Rodgers, R., Zhao, G., Sullender, M., Peterson, S.T., Locke, M., Liu, T.C., Yokoyama, C.C., Sharp, B., Schultz-Cherry, S., Miner, J.J., Baldridge, M.T. (2019). Viral complementation of immunodeficiency confers protection against enteric pathogens via interferon-λ. Nat. Microbiol. 4, 1120-1128. https://doi.org/10.1038/s41564-019-0416-7.

Jiang, B., Monroe, S.S., Koonin, E.V., Stine, S.E., Glass, R.I. (1993). RNA sequence of astrovirus: distinctive genomic organization and a putative retrovirus-like ribosomal frameshifting signal that directs the viral replicase synthesis. Proc. Natl. Acad. Sci. USA. 90, 10539-10543. https://doi.org/10.1073/pnas.90.22.10539.

Johne, R., Dremsek, P., Kindler, E., Schielke, A., Plenge-Bönig, A., Gregersen, H., Wessels, U., Schmidt, K., Rietschel, W., Groschup, M.H., Guenther, S., Heckel, G., Ulrich, R.G. (2012). Rat hepatitis E virus: geographical clustering within Germany and serological detection in wild Norway rats (Rattus norvegicus). Infect. Genet. Evol. 12, 947-956. https://doi.org/10.1016/j.meegid.2012.02.021.

Jonassen, C.M., Jonassen, T.Ø., Saif, Y.M., Snodgrass, D.R, Ushijima, H., Shimizu, M., Grinde, B. (2001). Comparison of capsid sequences from human and animal astroviruses. J. Gen. Virol. 82, 1061-1067. https://doi.org/10.1099/0022-1317-82-5-1061.

Kabrane-Lazizi, Y., Meng, X.J., Purcell, R.H., Emerson, S.U. (1999). Evidence that the genomic RNA of hepatitis E virus is capped. J. Virol. 73, 8848-8850. https://doi.org/10.1128/JVI.73.10.8848-8850.1999.

Kapoor, A., Li, L., Victoria, J., Oderinde, B., Mason, C., Pandey, P., Zaidi, S.Z., Delwart, E. (2009). Multiple novel astrovirus species in human stool. J. Gen. Virol. 90, 2965-2972. https://doi.org/10.1099/vir.0.014449-0.

Karst, S.M., Wobus, C.E., Lay, M., Davidson, J., Virgin, H.W. (2003). STAT1-dependent innate immunity to a Norwalk-like virus. Science. 299, 1575-1578. https://doi.org/10.1126/science.1077905.

Kelly, A.G., Netzler, N.E., White, P.A. (2016). Ancient recombination events and the origins of hepatitis E virus. BMC. Evol. Biol. 16, 210. https://doi.org/10.1186/s12862-016-0785-y.

Khuroo, M.S. (2011). Discovery of hepatitis E: the epidemic non-A, non-B hepatitis 30 years down the memory lane. Virus. Res. 161, 3-14. https://doi.org/10.1016/j.virusres.2011.02.007.

Kiang, D., Matsui, S.M. (2002). Proteolytic processing of a human astrovirus nonstructural protein. J. Gen. Virol. 83, 25-34. https://doi.org/10.1099/0022-1317-83-1-25.

Kjeldsberg, E., Hem, A. (1985). Detection of astroviruses in gut contents of nude and normal mice. Arch. Virol. 84, 135-140. https://doi.org/10.1007/BF01310560.

Koonin, E.V. (1991). The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. J. Gen. Virol. 72, 2197-2206. https://doi.org/10.1099/0022-1317-72-9-2197.

Koonin, E.V., Wolf, Y.I., Nagasaki, K., Dolja, V.V. (2008). The Big Bang of picorna-like virus evolution antedates the radiation of eukaryotic supergroups. Nat. Rev. Microbiol. 6, 925-939. https://doi.org/10.1038/nrmicro2030.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol. Biol. Evol. 35, 1547-1549. https://doi.org/10.1093/molbev/msy096.

Lateef, Z., Gimenez, G., Baker, E.S., Ward, V.K. (2017). Transcriptomic analysis of human norovirus NS1-2 protein highlights a multifunctional role in murine monocytes. BMC. Genomics. 18, 39. https://doi.org/10.1186/s12864-016-3417-4.

Liu, B., Clarke, I.N., Lambden, P.R. (1996). Polyprotein processing in Southampton virus: identification of 3C-like protease cleavage sites by in vitro mutagenesis. J. Virol. 70, 2605-2610. https://doi.org/10.1128/JVI.70.4.2605-2610.1996.

Liu, Y.G., Chen, Y. (2007). High-efficiency thermal asymmetric interlaced PCR for amplification of unknown flanking sequences. Biotechniques. 43, 649-650. https://doi.org/10.2144/000112601.

Livingston, R.S., Riley, L.K. (2003). Diagnostic testing of mouse and rat colonies for infectious agents. Lab. Anim. 32, 44-51. https://doi.org/10.1038/laban0503-44.

Lole, K.S., Bollinger, R.C., Paranjape, R.S., Gadkari, D., Kulkarni, S.S., Novak, N.G., Ingersoll, R., Sheppard, H.W., Ray, S.C. (1999). Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J. Virol. 73, 152-160. https://doi.org/10.1128/JVI.73.1.152-160.1999.

McFadden, N., Bailey, D., Carrara, G., Benson, A., Chaudhry, Y., Shortland, A., Heeney, J., Yarovinsky, F., Simmonds, P., Macdonald, A., Goodfellow, I. (2011). Norovirus regulation of the innate immune response and apoptosis occurs via the product of the alternative open reading frame 4. PLoS. Pathog. 7, e1002413. https://doi.org/10.1371/journal.ppat.1002413.

McFarlane, R., Sleigh, A., McMichael, T. (2012). Synanthropy of wild mammals as a determinant of emerging infectious diseases in the Asian-Australasian region. Ecohealth. 9, 24-35. https://doi.org/10.1007/s10393-012-0763-9.

Meerburg, B.G., Singleton, G.R., Kijlstra, A. (2009). Rodent-borne diseases and their risks for public health. Crit. Rev. Microbiol. 35, 221-270. https://doi.org/10.1080/10408410902989837.

Méndez, E., Murillo, A., Velázquez, R., Burnham, A., Arias, C.F. (2013). Chapter 2 – Replication cycle of astroviruses. in: Schultz-Cherry, S., (Ed.), Astrovirus research, Essential Ideas, Everyday Impacts, Future directions. Springer Science + Business Media, New York, pp. 19-47. https://doi.org/10.1007/978-1-4614-4735-1. ISBN: 978-1-4614-4734-4.

Méndez, E., Salas-Ocampo, E., Arias, C.F. (2004). Caspases mediate processing of the capsid precursor and cell release of human astroviruses. J. Virol. 78, 8601-8608. https://doi.org/10.1128/JVI.78.16.8601-8608.2004.

Monceyron, C., Grinde, B., Jonassen, T.O. (1997). Molecular characterisation of the 3'-end of the astrovirus genome. Arch. Virol. 142, 699-706. https://doi.org/10.1007/s007050050112.

Moreira, J.C.O., Rodrigues, D.M., Gilioli, R., Costa, F.F., Albuquerque, D.M. (2019). A semi-nested RT-PCR assay for detection of norovirus in rat fecal samples. Exp. Anim. 68, 169-176. https://doi.org/10.1538/expanim.18-0049.

Morse, S.S. (1995). Factors in the emergence of infectious diseases. Emerg. Infect. Dis. 1, 7-15. https://doi.org/10.3201/eid0101.950102.

Mulyanto, Suparyatmo, J.B., Andayani, I.G., Khalid, Takahashi, M., Ohnishi, H., Jirintai, S., Nagashima, S., Nishizawa, T., Okamoto, H. (2013). Marked genomic heterogeneity of rat hepatitis E virus strains in Indonesia demonstrated on a full-length genome analysis. Virus. Res. 179, 102-112. https://doi.org/10.1016/j.virusres.2013.10.029.

Ng, K.K., Pendás-Franco, N., Rojo, J., Boga, J.A., Machín, A., Alonso, J.M., Parra, F. (2004). Crystal structure of norwalk virus polymerase reveals the carboxyl terminus in the active site cleft. J. Biol. Chem. 279, 16638-16645. https://doi.org/10.1074/jbc.M400584200.

Ng, T.F., Kondov, N.O., Hayashimoto, N., Uchida, R., Cha, Y., Beyer, A.I., Wong, W., Pesavento, P.A., Suemizu, H., Muench, M.O., Delwart, E. (2013). Identification of an astrovirus commonly infecting laboratory mice in the US and Japan. PLoS One. 8, e66937. https://doi.org/10.1371/journal.pone.0066937.

Nguyen Ba, A.N., Pogoutse, A., Provart, N., Moses, A.M. (2009). NLStradamus: a simple Hidden Markov Model for nuclear localization signal prediction. BMC. Bioinformatics. 10, 202. https://doi.org/10.1186/1471-2105-10-202.

Nice, T.J., Baldridge, M.T., McCune, B.T., Norman, J.M., Lazear, H.M., Artyomov, M., Diamond, M.S., Virgin, H.W. (2015). Interferon-λ cures persistent murine norovirus infection in the absence of adaptive immunity. Science. 347, 269-273. https://doi.org/10.1126/science.1258100.

Nicolas, K.B., Nicolas, H.B. (1997). GeneDoc: a tool for editing and annotating multiple sequence alignments. www.psc.edu/biomed/genedoc

Omasits, U., Ahrens, C.H., Müller, S., Wollscheid, B. (2013). Protter: interactive protein feature visualization and integration with experimental proteomic data. Bioinformatics. 30, 884-886. https://doi.org/10.1093/bioinformatics/btt607.

Pfister, T., Wimmer, E. (2001). Polypeptide p41 of a Norwalk-like virus is a nucleic acid-independent nucleoside triphosphatase. J. Virol. 75, 1611-1619. https://doi.org/10.1128/JVI.75.4.1611-1619.2001.

Phan, T.G., Vo, N.P., Boros, Á., Pankovics, P., Reuter, G., Li, O.T., Wang, C., Deng, X., Poon, L.L., Delwart, E. (2013). The viruses of wild pigeon droppings. PLoS One. 8, e72787. https://doi.org/10.1371/journal.pone.0072787.

Potter, S.C., Luciani, A., Eddy, S.R., Park, Y., Lopez, R., Finn, R.D. (2018). HMMER web server: 2018 update. Nucleic. Acids. Res. 46, W200-W204. https://doi.org/10.1093/nar/gky448.

Prilusky, J., Felder, C.E., Zeev-Ben-Mordehai, T., Rydberg, E.H., Man, O., Beckmann, J.S., Silman, I., Sussman, J.L. (2005). FoldIndex: a simple tool to predict whether a given protein sequence is intrinsically unfolded. Bioinformatics. 21, 3435-3438. https://doi.org/10.1093/bioinformatics/bti537.

Primadharsini, P.P., Nagashima, S., Okamoto, H. (2019). Genetic Variability and Evolution of Hepatitis E Virus. Viruses. 11, 456. https://doi.org/10.3390/v11050456.

Reuter, G., Boros, Á., Pankovics, P. (2020). Review of Hepatitis E Virus in Rats: Evident Risk of Species Orthohepevirus C to Human Zoonotic Infection and Disease. Viruses. 12, 1148. https://doi.org/10.3390/v12101148. PMID: 33050353; PMCID: PMC7600399.

Sachsenröder, J., Braun, A., Machnowska, P., Ng, T.F.F., Deng, X., Guenther, S., Bernstein, S., Ulrich, R.G., Delwart, E., Johne, R. (2014). Metagenomic identification of novel enteric viruses in urban wild rats and genome characterization of a group A rotavirus. J. Gen. Virol. 95, 2734-2747. https://doi.org/10.1099/vir.0.070029-0.

Schlosser, B., Stein, A., Neuhaus, R., Pahl, S., Ramez, B., Krüger, D.H., Berg, T., Hofmann, J. (2012). Liver transplant from a donor with occult HEV infection induced chronic hepatitis and cirrhosis in the recipient. J. Hepatol. 56, 500-502. https://doi.org/10.1016/j.jhep.2011.06.021.

Shi, M., Lin, X.D., Tian, J.H., Chen, L.J., Chen, X., Li, C.X., Qin, X.C., Li, J., Cao, J.P., Eden, J.S., Buchmann, J., Wang, W., Xu, J., Holmes, E.C., Zhang, Y.Z. (2016). Redefining the invertebrate RNA virosphere. Nature. 540, 539-543. https://doi.org/10.1038/nature20167.

Shi, M., Zhang, Y.Z., Holmes, E.C. (2018). Meta-transcriptomics and the evolutionary biology of RNA viruses. Virus. Res. 243, 83-90. https://doi.org/10.1016/j.virusres.2017.10.016.

Smith, D.B., Simmonds, P., Jameel, S., Emerson, S.U., Harrison, T.J., Meng, X.J., Okamoto, H., Van der Poel, W.H.M., Purdy, M.A. (2014). Consensus proposals for classification of the family Hepeviridae. J. Gen. Virol. 95, 2223-2232. https://doi.org/10.1099/vir.0.068429-0.

Someya, Y., Takeda, N., Miyamura, T. (2002). Identification of active-site amino acid residues in the Chiba virus 3C-like protease. J. Virol. 76, 5949-5958. https://doi.org/10.1128/jvi.76.12.5949-5958.2002.

Sosnovtsev, S.V., Belliot, G., Chang, K.O., Prikhodko, V.G., Thackray, L.B., Wobus, C.E., Karst, S.M., Virgin, H.W., Green, K.Y. (2006). Cleavage map and proteolytic processing of the murine norovirus nonstructural polyprotein in infected cells. J. Virol. 80, 7816-7831. https://doi.org/10.1128/JVI.00532-06.

Sridhar, S., Yip, C.C.Y., Wu, S., Cai, J., Zhang, A.J., Leung, K.H., Chung, T.W.H., Chan, J.F.W., Chan, W.M., Teng, J.L.L., Au-Yeung, R.K.H., Cheng, V.C.C., Chen, H., Lau, S.K.P., Woo, P.C.Y., Xia, N.S., Lo, C.M., Yuen, K.Y. (2018). Rat Hepatitis E Virus as Cause of Persistent Hepatitis after Liver Transplant. Emerg. Infect. Dis. 24, 2241-2250. https://doi.org/10.3201/eid2412.180937.

Sverdlov, E. Azhikina, T. (2005). Primer walking. in: Maccarrone, M. (Ed.), eLS. John Wiley & Sons Ltd, Chichester. http://www.els.net. https://doi.org/10.1038/npg.els.0005382.

Takahashi, M., Kobayashi, T., Tanggis, Jirintai, S., Mulyanto, Nagashima, S., Nishizawa, T., Kunita, S., Okamoto, H. (2016). Production of monoclonal antibodies against the ORF3 protein of rat hepatitis E virus (HEV) and demonstration of the incorporation of the ORF3 protein into enveloped rat HEV particles. Arch. Virol. 161, 3391-3404. https://doi.org/10.1007/s00705-016-3047-9.

Tanggis, Kobayashi, T., Takahashi, M., Jirintai, S., Nishizawa, T., Nagashima, S., Nishiyama, T., Kunita, S., Hayama, E., Tanaka, T., Mulyanto, Okamoto, H. (2018). An analysis of two open reading frames (ORF3 and ORF4) of rat hepatitis E virus genome using its infectious cDNA clones with mutations in ORF3 or ORF4. Virus. Res. 249, 16-30. https://doi.org/10.1016/j.virusres.2018.02.014.

Tengs, T., Kristoffersen, A.B., Bachvaroff, T.R., Jonassen, C.M. (2013). A mobile genetic element with unknown function found in distantly related viruses. Virol. J. 10, 132. https://doi.org/10.1186/1743-422X-10-132.

To, K.K.W., Chan, W.M., Li, K.S.M., Lam, C.S.F., Chen, Z., Tse, H., Lau, S.K.P., Woo, P.C.Y., Yuen, K.Y. (2017). High prevalence of four novel astrovirus genotype species identified from rodents in China. J. Gen. Virol. 98, 1004-1015. https://doi.org/10.1099/jgv.0.000766.

Trifinopoulos, J., Nguyen, L.T., von Haeseler, A., Minh, B.Q. (2016). W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. Nucleic. Acids. Res. 44, W232-W235. https://doi.org/10.1093/nar/gkw256.

Tse, H., Chan, W.M., Lam, C.S., Lau, S.K., Woo, P.C., Yuen, K.Y. (2012). Complete genome sequences of novel rat noroviruses in Hong Kong. J. Virol. 86, 12435-12436. https://doi.org/10.1128/JVI.01976-12.

Tsunesumi, N., Sato, G., Iwasa, M., Kabeya, H., Maruyama, S., Tohya, Y. (2012). Novel murine norovirus-like genes in wild rodents in Japan. J. Vet. Med. Sci. 74, 1221-1224. https://doi.org/10.1292/jvms.12-0106.

Vinjé, J., Estes, M.K., Esteves, P., Green, K.Y., Katayama, K., Knowles, N.J., L'Homme, Y., Martella, V., Vennema, H., White, P.A. (2019). ICTV Virus Taxonomy Profile: Caliciviridae. J. Gen. Virol. 100, 1469-1470. https://doi.org/10.1099/jgv.0.001332.

Wang, H., Zhang, W., Gu, H., Chen, W., Zeng, M., Ji, C., Song, R., Zhang, G. (2017). Identification and characterization of two linear epitope motifs in hepatitis E virus ORF2 protein. PLoS One. 12, e0184947. https://doi.org/10.1371/journal.pone.0184947.

Wobus, C.E., Karst, S.M., Thackray, L.B., Chang, K.O., Sosnovtsev, S.V., Belliot, G., Krug, A., Mackenzie, J.M., Green, K.Y., Virgin, H.W. (2004). Replication of Norovirus in cell culture reveals a tropism for dendritic cells and macrophages. PLoS Biol. 2, e432. https://doi.org/10.1371/journal.pbio.0020432.

Wolf, S., Reetz, J., Johne, R., Heiberg, A.C., Petri, S., Kanig, H., Ulrich, R.G. (2013). The simultaneous occurrence of human norovirus and hepatitis E virus in a Norway rat (Rattus norvegicus). Arch. Virol. 158, 1575-1578. https://doi.org/10.1007/s00705-013-1646-2.

Wu, Z., Yang, L., Ren, X., He, G., Zhang, J., Yang, J., Qian, Z., Dong, J., Sun, L., Zhu, Y., Du, J., Yang, F., Zhang, S., Jin, Q. (2015). Deciphering the bat virome catalog to better understand the ecological diversity of bat viruses and the bat origin of emerging infectious diseases. ISME. J. 10, 609-620. https://doi.org/10.1038/ismej.2015.138.

Xie, X., Wang, H., Zeng, J., Li, C., Zhou, G., Yang, D., Yu, L. (2014). Foot-and-mouth disease virus low-fidelity polymerase mutants are attenuated. Arch. Virol. 159, 2641-2650. https://doi.org/10.1007/s00705-014-2126-z.

Yokoyama, C.C., Loh, J., Zhao, G., Stappenbeck, T.S., Wang, D., Huang, H.V., Virgin, H.W., Thackray, L.B. (2012). Adaptive immunity restricts replication of novel murine astroviruses. J. Virol. 86, 12262-12270. https://doi.org/10.1128/JVI.02018-12.

Yom-Tov, Y. (1985). The reproductive rates of Australian rodents. Oecologia. 66, 250-255. https://doi.org/10.1007/BF00379862.

Zhou, N., Lin, X., Wang, S., Wang, H., Bi, Z., Wang, P., Chen, P., Tao, Z., Song, L., Song, Y., Xu, A. (2016). Molecular characterization of classic human astrovirus in eastern China, as revealed by environmental sewage surveillance. J. Appl. Microbiol. 120, 1436-1444. https://doi.org/10.1111/jam.13109.

Zhou, N., Lin, X., Wang, S., Wang, H., Li, W., Tao, Z., Xu, A. (2013). Environmental surveillance for human astrovirus in Shandong Province, China in 2013. Sci. Rep. 4, 7539. https://doi.org/10.1038/srep07539.

Web references

Animal Diversity Web. University of Michigan, Museum of Zoology, https://animaldiversity.org/accounts/Rodentia/ (accessed in 16.10.2020), https://animaldiversity.org/accounts/Muridae/ (accessed in 16.10.2020), https://animaldiversity.org/accounts/Rattus_norvegicus/ (accessed in 16.10.2020).

ViralZone. SIB Swiss Institute of Bioinformatics, https://viralzone.expasy.org/ (accessed in 16.10.2020)

Virus Taxonomy: 2019 Release, ICTV Online: Norovirus, https://talk.ictvonline.org/ictv-reports/ictv_online_report/positive-sense-rna-viruses/w/caliciviridae/1167/genus-norovirus (accessed in 16.11.2020)

Virus Taxonomy: 2019 Release, ICTV Online: *Hepeviridae*, https://talk.ictvonline.org/ictv-reports/ictv_online_report/positive-sense-rna-viruses/w/hepeviridae (accessed in 16.11.2020)

Figure legends

Figure 1. Comparative genome organization of (**A**) astrovirus, (**B**) hepevirus and (**C**) norovirus from faecal sample of Norway rat (*Rattus norvegicus*). The RNA genome of the (**A**) astrovirus strain NR/rat01-AstV/HUN/2018, (**B**) hepevirus strain NR/rat08-HEV/HUN/2018 and (**C**) norovirus strain NR/rat01-NoV/HUN/2018 was compared by SimPlot software (Lole et al., 1999) after alignment with the closest related sequence of rodent astrovirus strain HK-1797F (KT946732), rat hepevirus strain LA-B350 (KM516906) and rat norovirus strain Rn/GV/HKU_KT/HKG/2012 (JX486102), respectively. The same settings (window: 200bp; step: 50bp; gap-strip: on) and F84 parameters were used for all three genome comparisons showing the genome length (X-axis) and the similarity identity score (Y-axis). The overall percent identity scores to the reference sequence, the nucleotide start and stop position of the potential open reading frames (ORFs) and the length of each ORFs are highlighted in the figure. The conserved nucleotide (nt) and amino acid (aa) motifs are indicated to the schematic genome organization of each ORFs. **A**) Both, the conserved frameshift signal (FS) and the subgenomic RNA promoter (SG) are identifiable in the rat astrovirus genome. The coiled-coiled (CC) elements; the transmembrane (TM) domains; the trypsin-like protease (TLP); the nuclear localization signal (NLS) and the viral protein genome-linked (VPg) highlighting the TEQEY motif are also present in the encoded genome of ORF1a. The RNA-dependent RNA polymerase (RdRp) in the ORF1b and viral capsid protein in ORF2 are also identifiable in the genome. **B**) The conserved cis-acting replication element (CRE) and the transcription start site (TSS) are identifiable in the rat hepevirus genome. The methyltransferase domain (MT-Y): the papain-like cysteine protease domain (PCP); the hypervariable region (HVR); the poly-proline region (PPR); the macro domain (X); the helicase enzyme (Hel); the RNA-dependent RNA polymerase (RdRp) is detectable in ORF1, The S-, P1- and P2 domains of ORF2-encoded viral capsid protein and the two main domains with the potential immunodominant region in ORF3-encoded

protein are also identifiable in rat hepevirus. For rat hepevirus strain NR/rat08-HEV/HUN/2018, the aa motifs identified in each domain were grouped, which are detailed as follows: motif_I: HPIQRTIHN, RDMQRW, SAGYSHDRKSIRSWI; motif_II: CGCFM; motif_III: VFRG, GPYWADVPP, SLQS, NPPVQP, RVLLEL; motif_IV: LVNAAN, GGGVCGA, GSVAAY, IIHAVAP, LQVAYC, AYCLLGSGIY, LVVTPGI; motif_V: RFVAGVPGSGKS, DLVIVPTNQL, KRLVTDPAAT, VVLLGDPKQ, QSHRCP, TVHEAQGSTFNTTAL, IVALTRHTDKC; motif_VI: KDCNKFTT, ISAWSKTLVALFGPWFRA, FYGDCYVQEKL, NDFSEFDSTQNN, KHSGEPGTLLWNTVW, AAFKGDDSIVC, SYAGLLVACG, TPDVVRFLGRLS; motif_VII: ADTLLGGLPTDLVSNA; motif_VIII: CVSLSCSCFCCSCRCCSRP, PSYPMP; motif_IX: LLS, GRIRYSALFIIFWRR, LLM, LLI.

C) The following non-structural peptides could be identified in the ORF1 region. NS1-2: the p48/N-terminal protein (Aichi virus in *Picornaviridae* 2A-like), PPR: disordered proline-rich region, TM: hydrophobic transmembrane domain (WSFA[…]IFW motif), H-box/NC: HYSI and NC motif, CC: coiled-coil structure between 451-474, CCS: caspase cleavage sites: DMSD*SAIF, DDDD*DDSQ, DDDD*DSQL, DDDD*SQLI, IERD*GKVR, EQDD*GPFY, DKKD*LMAT, DHWD*TYKG; NS3: the NTPase protein (picornavirus 2C-like), HC: hydrophobic consensus region (IVPTLLGGLGLALGM motif), AC: acidic consensus region (LDEEE motif), NTP-binding site: GRPGIGKT motif, SF3_motifs: classical SF3 helicase conserved motifs (WDDFG, TSNM, RRIDF), N_motifs: novel highly conserved motifs (RRRPV, DHWDTY, IENKGK); NS4: p22 protein (picornavirus 3A-like); NS5: viral protein linked to the genome with the TDEEY motif; NS6: the viral protease (picornavirus 3C -like), conserved amino acid motifs: GDCG and PAYLG; NS7: the viral RNA-dependent RNA polymerase (picornavirus 3D^{pol}-like), the active site of the protein at D_{1567}, conserved amino acid motifs: KDELV, GLPSG, YGDD, FLRR, KLDR; The viral capsid proteins could be

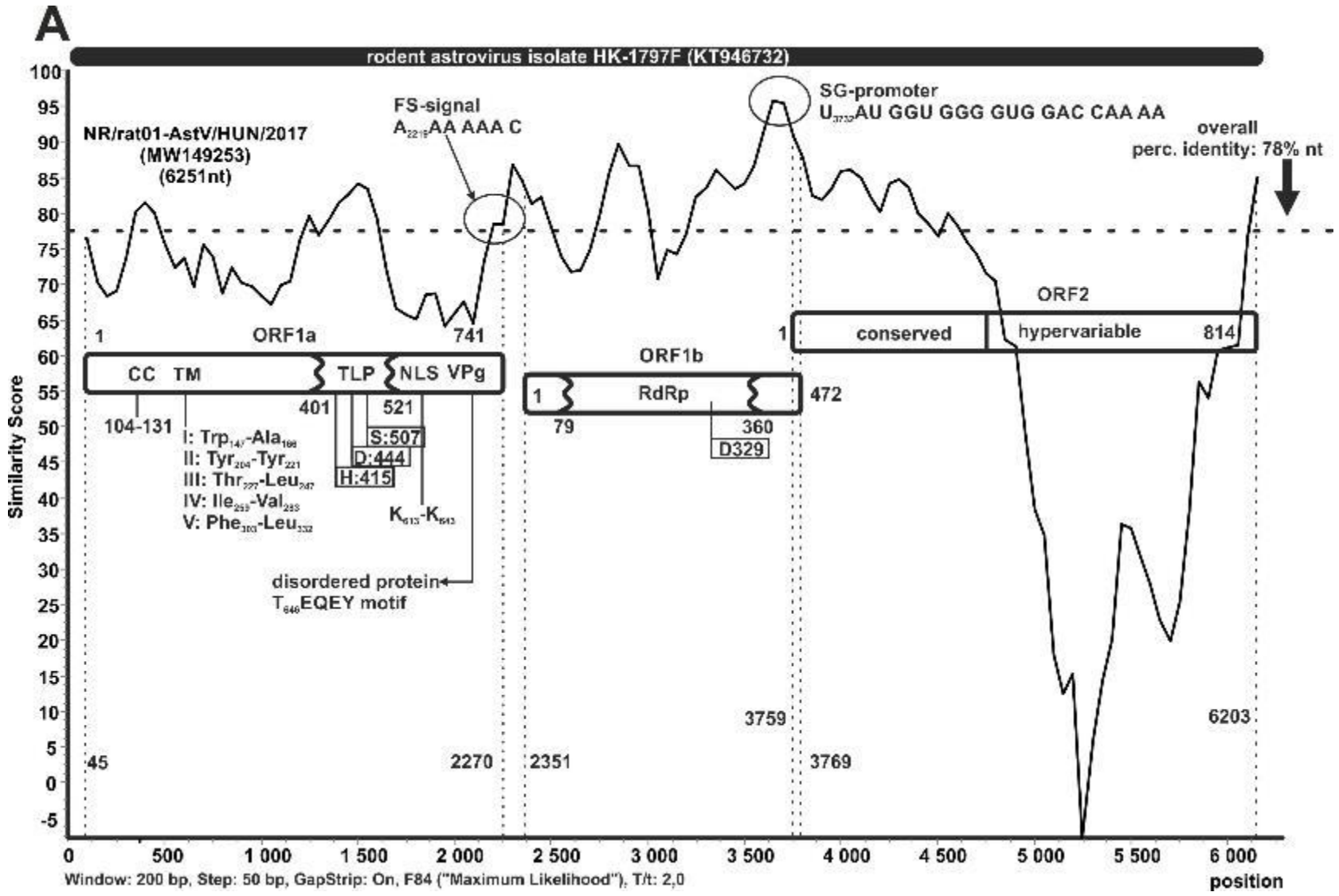
described in the open reading frame 2 and 3 (ORF2 and ORF3) regions. VP1: major viral capsid protein, VP2: minor viral capsid protein. The ORF4 could be detected in rat norovirus, VF: virulence factor 1

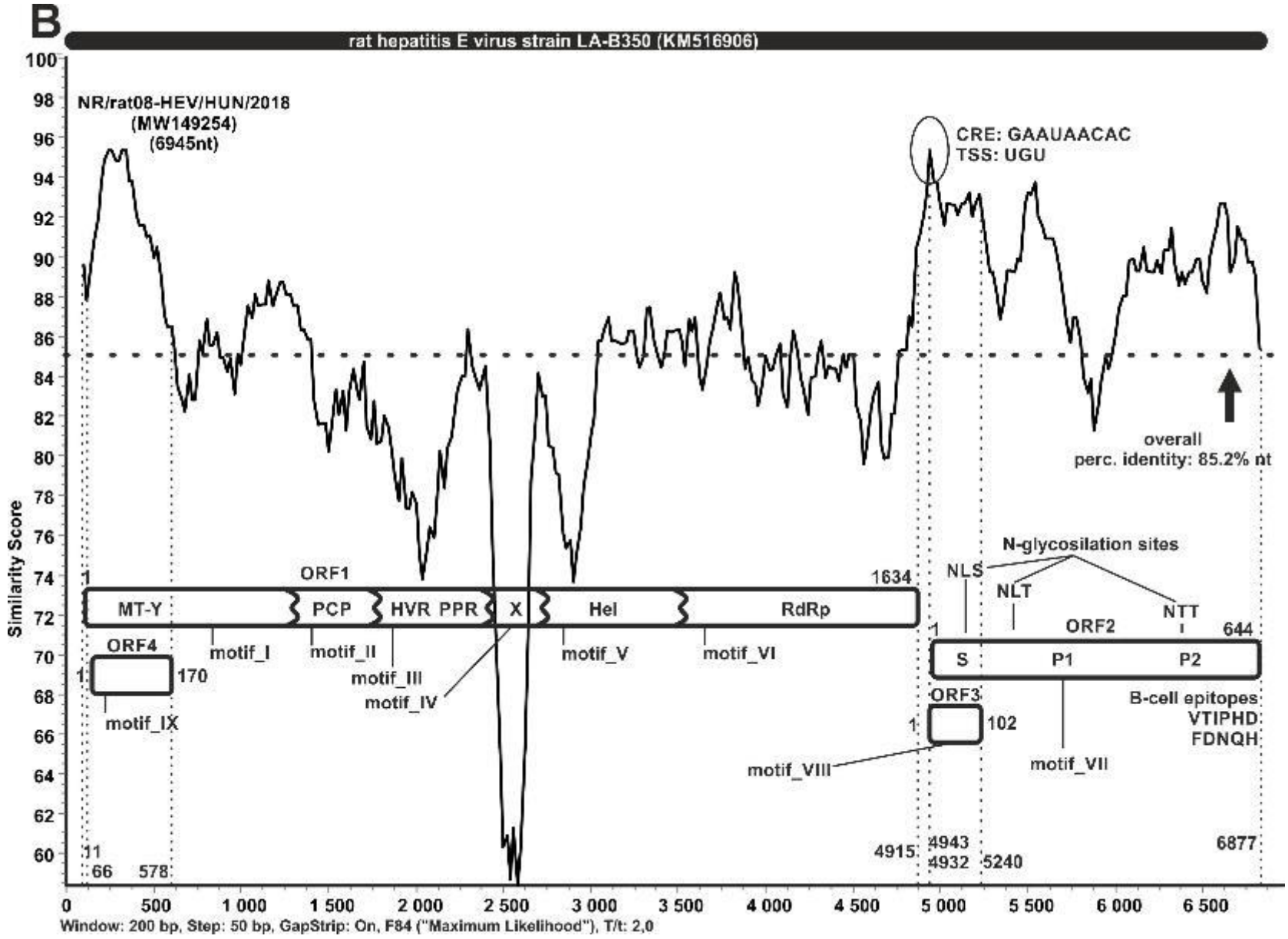
Figure 2. Phylogenetic analysis of three RNA viruses of free-living wild Norway rat (*Rattus norvegicus*) based on complete aa sequences open reading frames (ORFs) of study strains **A**) rat astrovirus strain NR/rat01-AstV/HUN/2017 (MW149253), **B**) rat hepevirus strain NR/rat08-HEV/HUN/2018 (MW149254) and **C**) rat norovirus strain NR/rat01-NoV/HUN/2017 (MW174170) and representative members of the aforementioned viruses. Dendrograms were constructed by the Maximum Likelihood method using MEGA (Kumar, Stecher, Li, Knyaz, and Tamura 2018) based on the JTT with Freqs (+F) model (Jones et al., 1992; Kumar et al., 2016) with bootstrap values and 1000 replicates. **A**) On the upper left the phylogenetic distribution of the reference sequences of family *Astroviridae* is presented, where murine and rodent viruses are marked by arrow and the study lineage is bolded by grey. The "cluster D" group of astroviruses named by To et al. (To et al., 2017) was indicated on the phylogenetic trees. **B**) On the upper left the phylogenetic distribution of the reference sequences of family *Caliciviridae* is presented, where GV murine noroviruses are marked by arrow and the study lineage is bolded by grey. **C**) On the upper left the phylogenetic distribution of the reference and complete genome sequences of family *Hepeviridae* is presented, where rodent viruses in *Orthohepevirus C1* are marked and the study lineage is bolded by grey.

Figure 3. The screening and quantitative determination of the study viruses from Norway rat (*Rattus norvegicus*) faecal and different organ samples. The origin of the test animals is indicated on the blind map of Hungary in the upper left corner of the figure (name of the settlement and the identifier of the collected sample). Animal IDs from this study: **rat01**-**rat10**,

HB1-**HB8**, **WP1**-**WP10**, **CSP1**-**CSP11**, **Rag1**-**Rag5**, **Nude1**-**Bude5**, **Bc1**-**Bc5** and **B6_1**-**B6-5**. The laboratory animals originated from Pécs are indicated with spotted lines. The results of the epidemiological study are shown in the upper right figure ("heat-map") in collection date, animal ID and sample type groups. Presentation of viruses detected in captured animals by screening RT-PCR technique: rat astrovirus (yellow), rat norovirus (green) and rat hepevirus (red) were identified from faeces, liver, spleen, kidney, lung, heart, muscle, brain and blood samples. Grey colour indicate unavailable specimens. The quantitative viral RNA determination of the study viruses using RT-qPCR technique from faecal or tissue samples are shown at the bottom of the figure. The amount of virus (virus copy) in each sample was determined from the equation of a known standard calibration series per unit mass (gram). Samples marked with an asterisk were RT-PCR positive, but virus quantification could not be determined.

Figure 1


A
rodent astrovirus isolate HK-1797F (KT946732)
NR/rat01-AstV/HUN/2017
(MW149253)
(6251nt)
FS-signal
A2218AA AAA C
SG-promoter
U3732AU GGU GGG GUG GAC CAA AA
overall
perc. identity: 78% nt
Similarity Score
ORF1a
1
741
CC TM
TLP
NLS VPg
401
521
104-131
I: Trp147-Ala166
II: Tyr204-Tyr221
III: Thr227-Leu247
IV: Ile253-Val283
V: Phe303-Leu332
S:507
D:444
H:415
K613-K643
disordered protein
T646EQEY motif
ORF1b
1
RdRp
472
79
360
D329
ORF2
1
conserved
hypervariable
814
45
2270
2351
3759
3769
6203
0
500
1 000
1 500
2 000
2 500
3 000
3 500
4 000
4 500
5 000
5 500
6 000
position
Window: 200 bp, Step: 50 bp, GapStrip: On, F84 ("Maximum Likelihood"), T/t: 2,0


B
rat hepatitis E virus strain LA-B350 (KM516906)
NR/rat08-HEV/HUN/2018
(MW149254)
(6945nt)
CRE: GAAUAACAC
TSS: UGU
overall
perc. identity: 85.2% nt
Similarity Score
ORF1
MT-Y
PCP
HVR
PPR
X
Hel
RdRp
motif_I
motif_II
motif_III
motif_IV
motif_V
motif_VI
ORF4
motif_IX
ORF2
S
P1
P2
N-glycosilation sites
NLS
NLT
NTT
ORF3
motif_VII
motif_VIII
B-cell epitopes
VTIPHD
FDNQH
Window: 200 bp, Step: 50 bp, GapStrip: On, F84 ("Maximum Likelihood"), T/t: 2,0

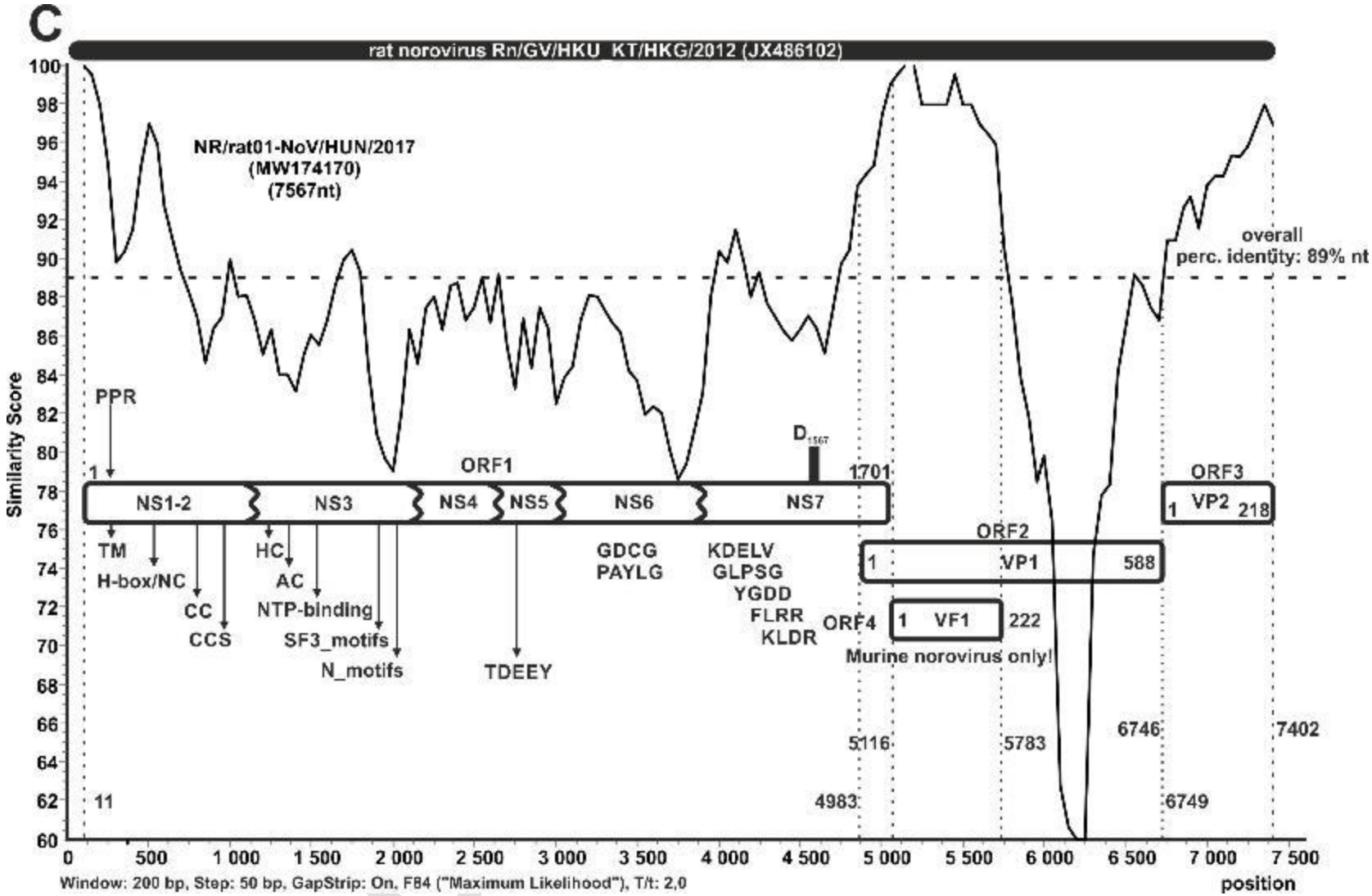
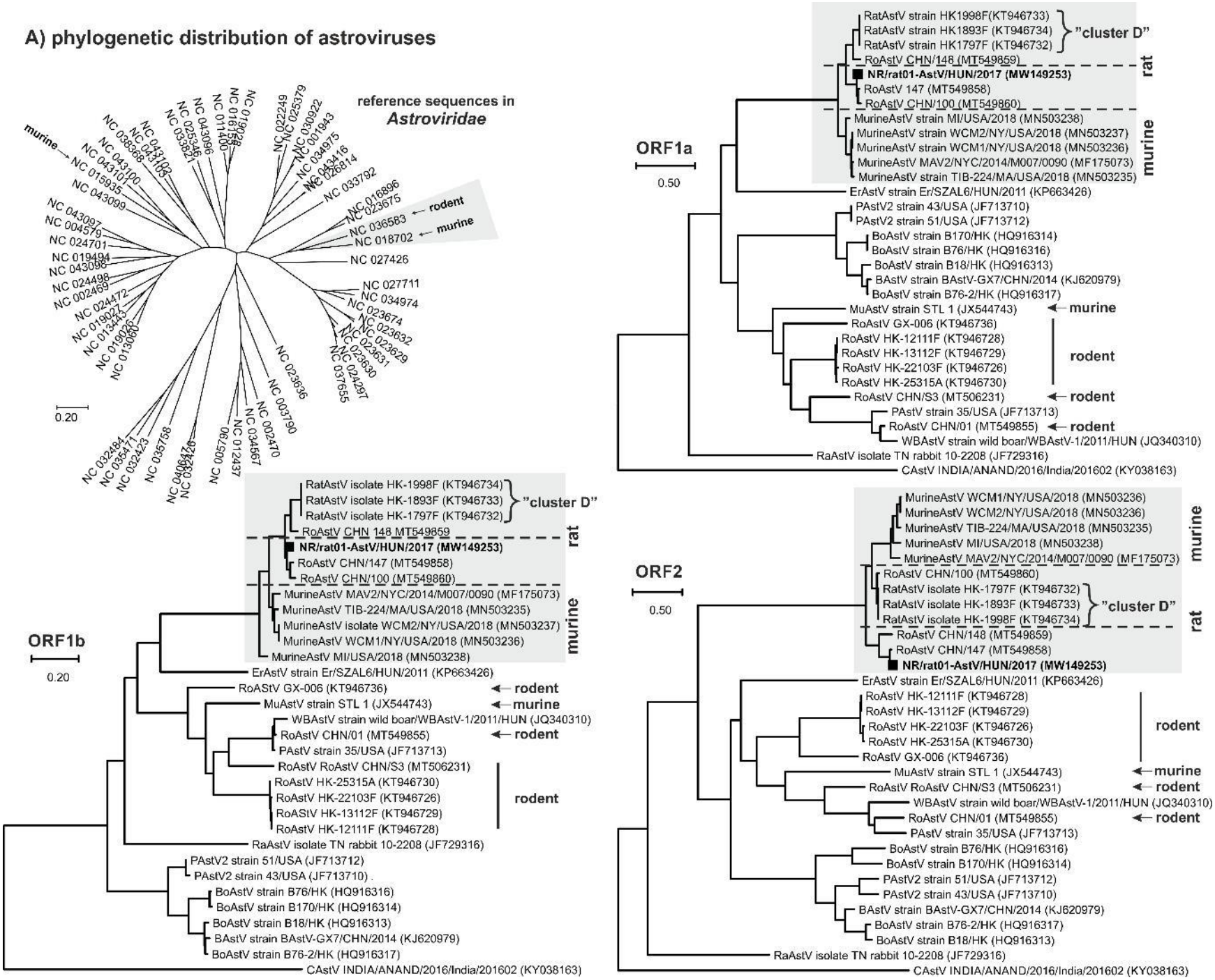
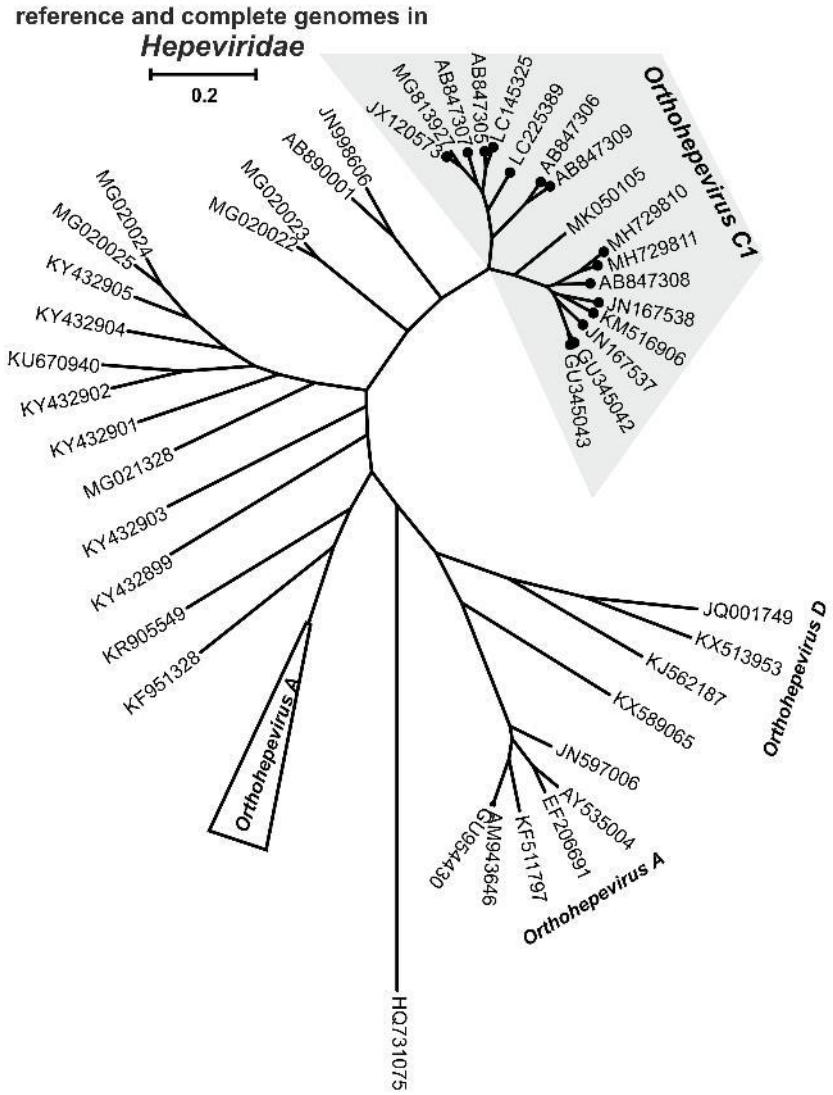
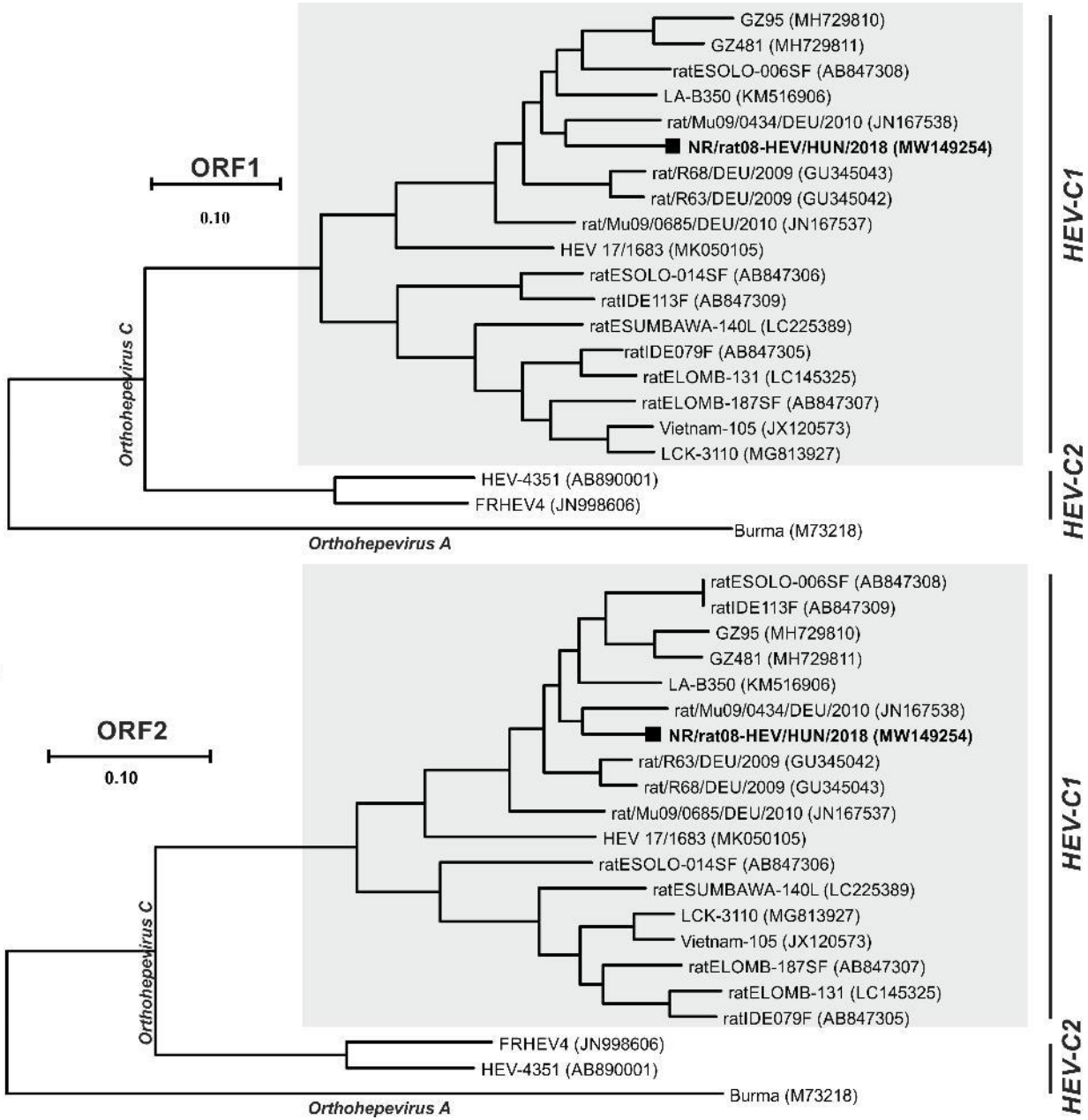

C
rat norovirus Rn/GV/HKU_KT/HKG/2012 (JX486102)
NR/rat01-NoV/HUN/2017
(MW174170)
(7567nt)
overall
perc. identity: 89% nt
Similarity Score
PPR
ORF1
NS1-2
NS3
NS4
NS5
NS6
NS7
TM
H-box/NC
CC
CCS
HC
AC
NTP-binding
SF3_motifs
N_motifs
TDEEY
GDCG
PAYLG
KDELV
GLPSG
YGDD
FLRR
KLDR
ORF2
VP1
ORF4
VF1
Murine norovirus only!
ORF3
VP2
11
4983
5116
5783
6746
6749
7402
Window: 200 bp, Step: 50 bp, GapStrip: On, F84 ("Maximum Likelihood"), T/t: 2,0
position

Figure 2.


A) phylogenetic distribution of astroviruses
reference sequences in
Astroviridae
murine
rodent
murine
0.20
ORF1b
0.20
RatAstV isolate HK-1998F (KT946734)
RatAstV isolate HK-1893F (KT946733)
RatAstV isolate HK-1797F (KT946732)
"cluster D"
RoAstV CHN 148 MT549859
NR/rat01-AstV/HUN/2017 (MW149253)
RoAstV CHN/147 (MT549858)
RoAstV CHN/100 (MT549860)
rat
MurineAstV MAV2/NYC/2014/M007/0090 (MF175073)
MurineAstV TIB-224/MA/USA/2018 (MN503235)
MurineAstV isolate WCM2/NY/USA/2018 (MN503237)
MurineAstV WCM1/NY/USA/2018 (MN503236)
MurineAstV MI/USA/2018 (MN503238)
murine
ErAstV strain Er/SZAL6/HUN/2011 (KP663426)
RoAStV GX-006 (KT946736)
rodent
MuAstV strain STL 1 (JX544743)
murine
WBAstV strain wild boar/WBAstV-1/2011/HUN (JQ340310)
RoAstV CHN/01 (MT549855)
rodent
PAstV strain 35/USA (JF713713)
RoAstV RoAstV CHN/S3 (MT506231)
RoAstV HK-25315A (KT946730)
RoAstV HK-22103F (KT946726)
RoAStV HK-13112F (KT946729)
RoAstV HK-12111F (KT946728)
rodent
RaAstV isolate TN rabbit 10-2208 (JF729316)
PAstV2 strain 51/USA (JF713712)
PAstV2 strain 43/USA (JF713710) .
BoAstV strain B76/HK (HQ916316)
BoAstV strain B170/HK (HQ916314)
BoAstV strain B18/HK (HQ916313)
BAstV strain BAstV-GX7/CHN/2014 (KJ620979)
BoAstV strain B76-2/HK (HQ916317)
CAstV INDIA/ANAND/2016/India/201602 (KY038163)
ORF1a
0.50
RatAstV strain HK1998F(KT946733)
RatAstV strain HK1893F (KT946734)
RatAstV strain HK1797F (KT946732)
"cluster D"
RoAstV CHN/148 (MT549859)
NR/rat01-AstV/HUN/2017 (MW149253)
RoAstV 147 (MT549858)
RoAstV CHN/100 (MT549860)
rat
MurineAstV strain MI/USA/2018 (MN503238)
MurineAstV strain WCM2/NY/USA/2018 (MN503237)
MurineAstV strain WCM1/NY/USA/2018 (MN503236)
MurineAstV MAV2/NYC/2014/M007/0090 (MF175073)
MurineAstV strain TIB-224/MA/USA/2018 (MN503235)
murine
ErAstV strain Er/SZAL6/HUN/2011 (KP663426)
PAstV2 strain 43/USA (JF713710)
PAstV2 strain 51/USA (JF713712)
BoAstV strain B170/HK (HQ916314)
BoAstV strain B76/HK (HQ916316)
BoAstV strain B18/HK (HQ916313)
BAstV strain BAstV-GX7/CHN/2014 (KJ620979)
BoAstV strain B76-2/HK (HQ916317)
MuAstV strain STL 1 (JX544743)
murine
RoAstV GX-006 (KT946736)
RoAstV HK-12111F (KT946728)
RoAstV HK-13112F (KT946729)
RoAstV HK-22103F (KT946726)
RoAstV HK-25315A (KT946730)
rodent
RoAstV CHN/S3 (MT506231)
rodent
PAstV strain 35/USA (JF713713)
RoAstV CHN/01 (MT549855)
rodent
WBAstV strain wild boar/WBAstV-1/2011/HUN (JQ340310)
RaAstV isolate TN rabbit 10-2208 (JF729316)
CAstV INDIA/ANAND/2016/India/201602 (KY038163)
ORF2
0.50
MurineAstV WCM1/NY/USA/2018 (MN503236)
MurineAstV WCM2/NY/USA/2018 (MN503236)
MurineAstV TIB-224/MA/USA/2018 (MN503235)
MurineAstV MI/USA/2018 (MN503238)
MurineAstV MAV2/NYC/2014/M007/0090 (MF175073)
murine
RoAstV CHN/100 (MT549860)
RatAstV isolate HK-1797F (KT946732)
RatAstV isolate HK-1893F (KT946733)
RatAstV isolate HK-1998F (KT946734)
"cluster D"
RoAstV CHN/148 (MT549859)
RoAstV CHN/147 (MT549858)
NR/rat01-AstV/HUN/2017 (MW149253)
rat
ErAstV strain Er/SZAL6/HUN/2011 (KP663426)
RoAstV HK-12111F (KT946728)
RoAstV HK-13112F (KT946729)
RoAstV HK-22103F (KT946726)
RoAstV HK-25315A (KT946730)
rodent
RoAstV GX-006 (KT946736)
MuAstV strain STL 1 (JX544743)
murine
RoAstV RoAstV CHN/S3 (MT506231)
rodent
WBAstV strain wild boar/WBAstV-1/2011/HUN (JQ340310)
RoAstV CHN/01 (MT549855)
rodent
PAstV strain 35/USA (JF713713)
BoAstV strain B76/HK (HQ916316)
BoAstV strain B170/HK (HQ916314)
PAstV2 strain 51/USA (JF713712)
PAstV2 strain 43/USA (JF713710)
BAstV strain BAstV-GX7/CHN/2014 (KJ620979)
BoAstV strain B76-2/HK (HQ916317)
BoAstV strain B18/HK (HQ916313)
RaAstV isolate TN rabbit 10-2208 (JF729316)
CAstV INDIA/ANAND/2016/India/201602 (KY038163)

B) phylogenetic distribution of hepeviruses





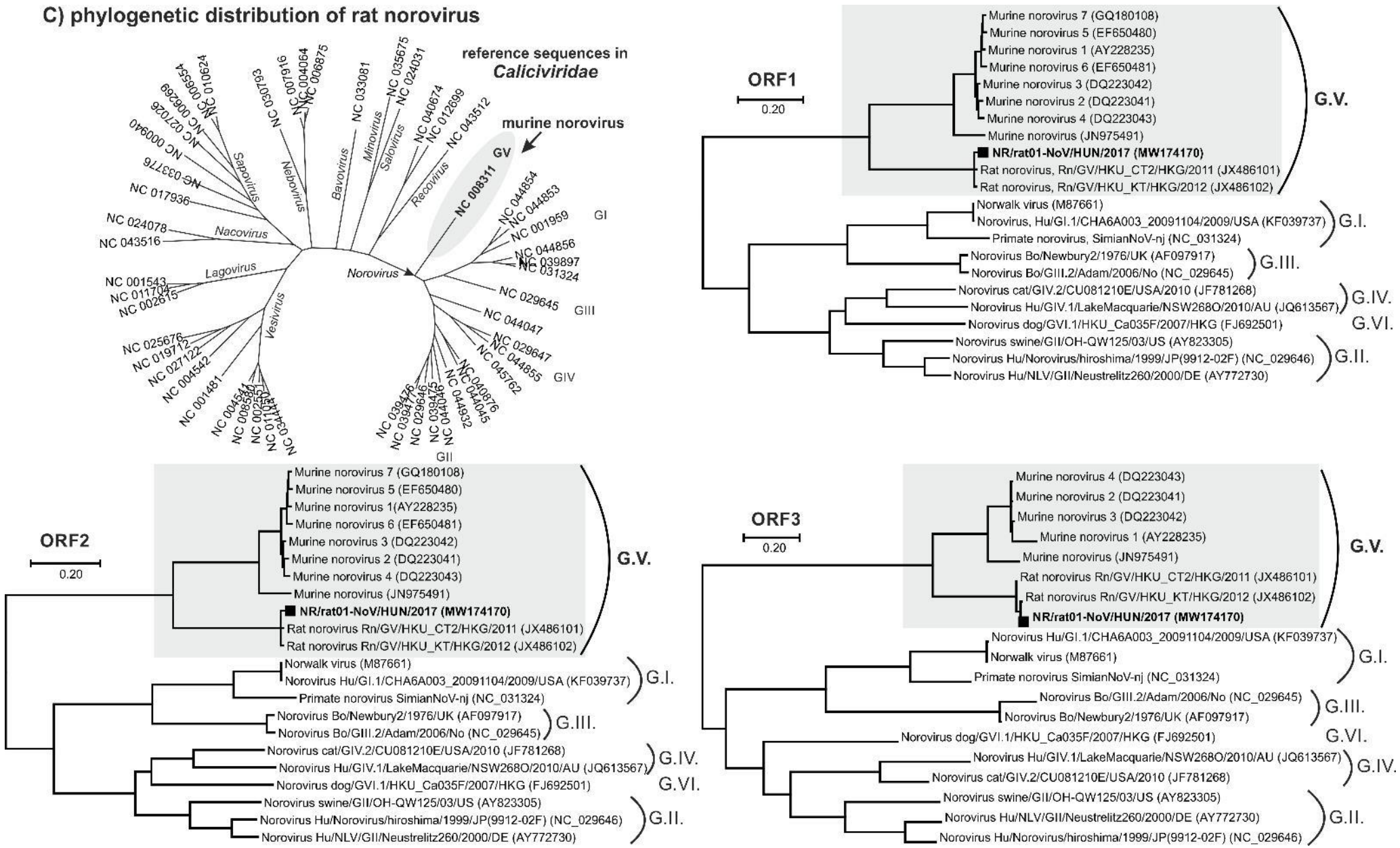
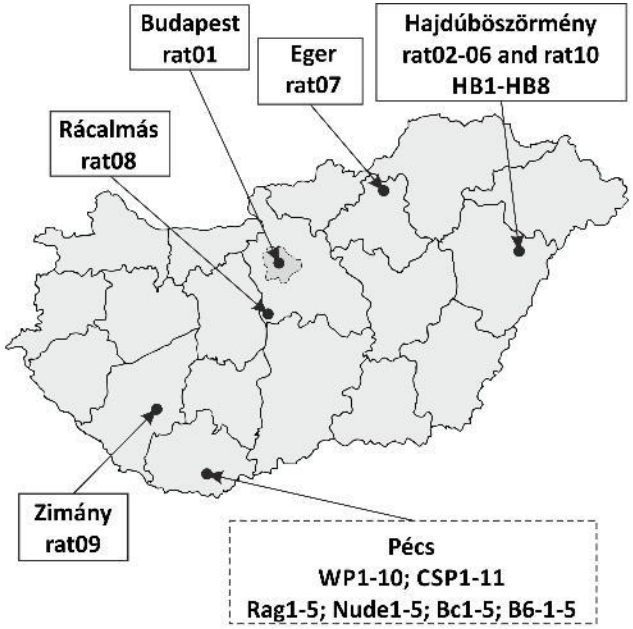
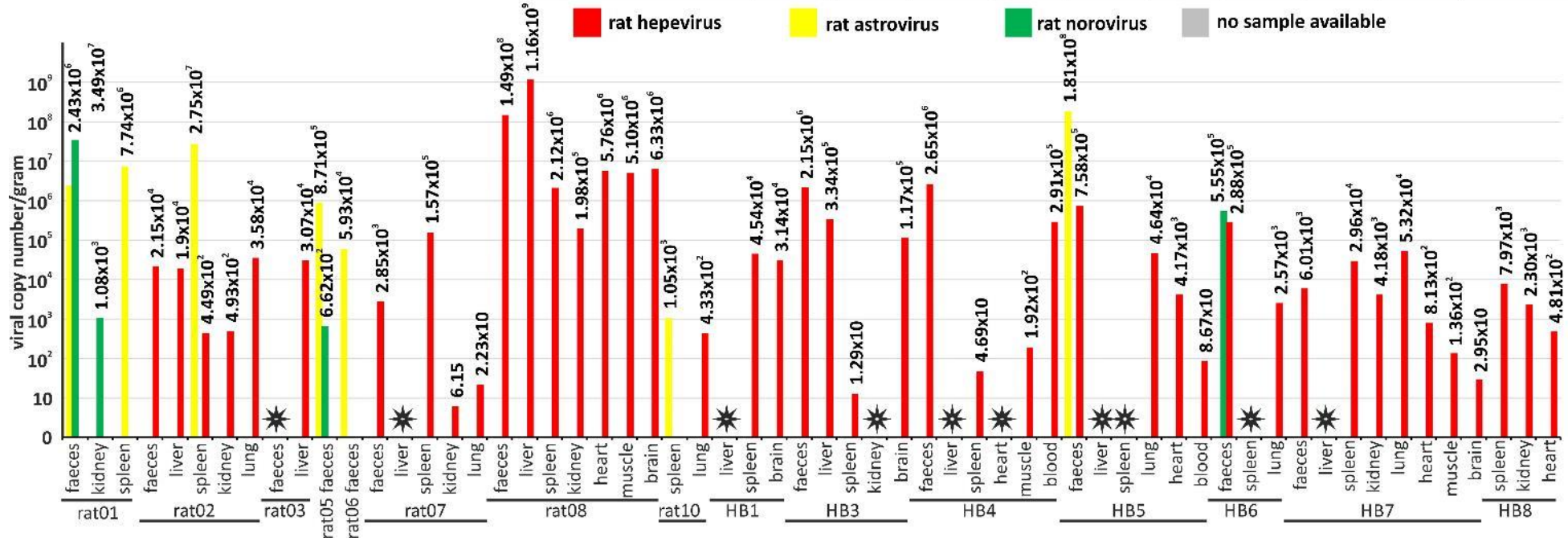

C) phylogenetic distribution of rat norovirus
reference sequences in
Caliciviridae
murine norovirus
GV
GI
GIII
GIV
GII
Sapovirus
Nebovirus
Bavovirus
Minovirus
Salovirus
Recovirus
Norovirus
Vesivirus
Lagovirus
Nacovirus
NC 008311
ORF1
0.20
Murine norovirus 7 (GQ180108)
Murine norovirus 5 (EF650480)
Murine norovirus 1 (AY228235)
Murine norovirus 6 (EF650481)
Murine norovirus 3 (DQ223042)
Murine norovirus 2 (DQ223041)
Murine norovirus 4 (DQ223043)
Murine norovirus (JN975491)
NR/rat01-NoV/HUN/2017 (MW174170)
Rat norovirus, Rn/GV/HKU_CT2/HKG/2011 (JX486101)
Rat norovirus, Rn/GV/HKU_KT/HKG/2012 (JX486102)
G.V.
Norwalk virus (M87661)
Norovirus, Hu/GI.1/CHA6A003_20091104/2009/USA (KF039737)
Primate norovirus, SimianNoV-nj (NC_031324)
G.I.
Norovirus Bo/Newbury2/1976/UK (AF097917)
Norovirus Bo/GIII.2/Adam/2006/No (NC_029645)
G.III.
Norovirus cat/GIV.2/CU081210E/USA/2010 (JF781268)
Norovirus Hu/GIV.1/LakeMacquarie/NSW268O/2010/AU (JQ613567)
G.IV.
Norovirus dog/GVI.1/HKU_Ca035F/2007/HKG (FJ692501)
G.VI.
Norovirus swine/GII/OH-QW125/03/US (AY823305)
Norovirus Hu/Norovirus/hiroshima/1999/JP(9912-02F) (NC_029646)
Norovirus Hu/NLV/GII/Neustrelitz260/2000/DE (AY772730)
G.II.
ORF2
0.20
ORF3
0.20

Figure 3

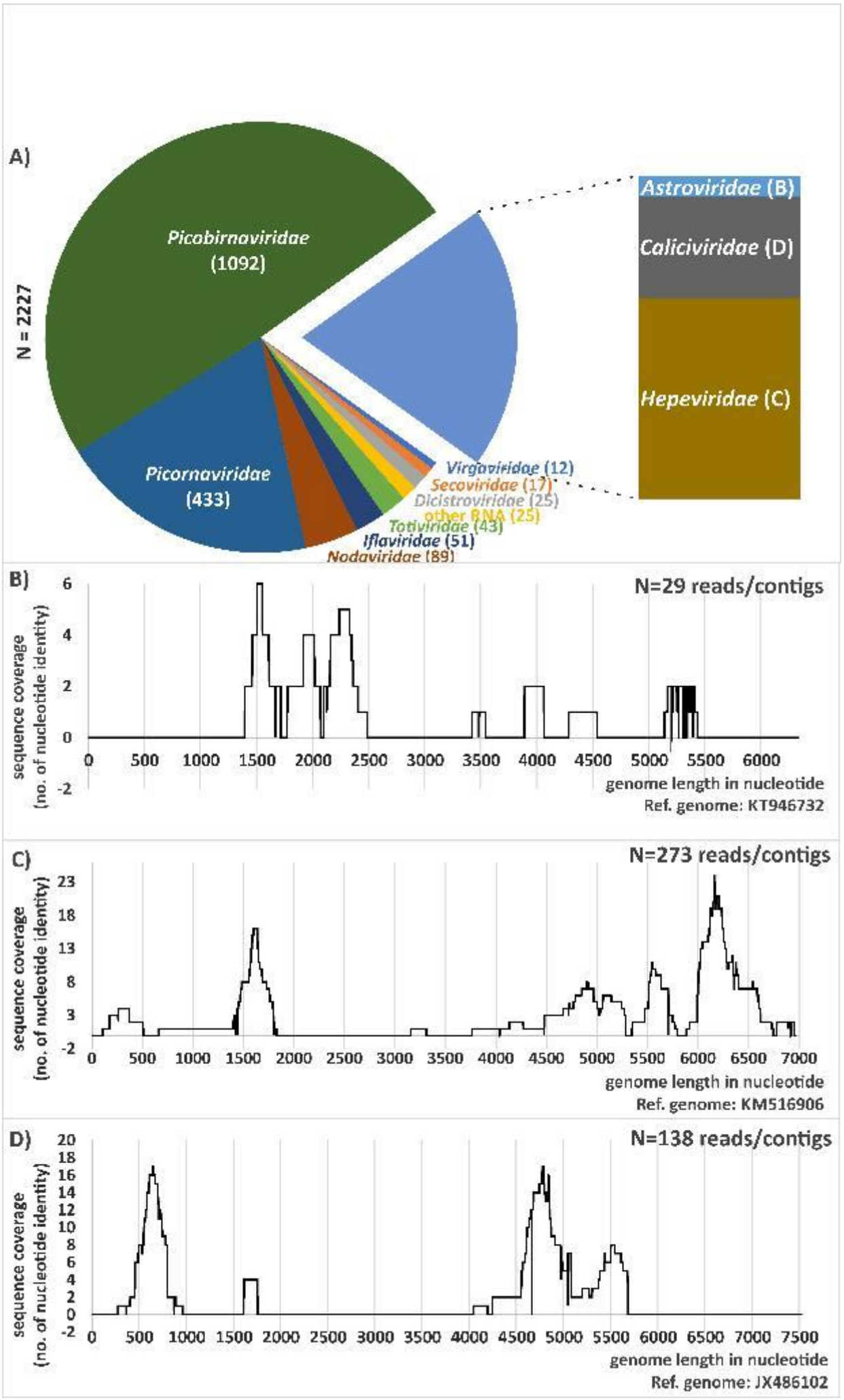


collection date	animal ID	faeces	liver	spleen	kidney	lung	heart	muscle	brain	blood	Total sample per animal
2017	rat01										5
	rat02										5
	rat03										2
	rat04										4
	rat05										2
	rat06										2
2018	rat07										5
	rat08										8
	rat09										4
	rat10										4
2019	HB1										8
	HB2										8
	HB3										9
	HB4										9
2020	HB5										9
	HB6										9
	HB7										8
	HB8										6
Total sample/type		17	18	15	14	13	10	8	8	4	



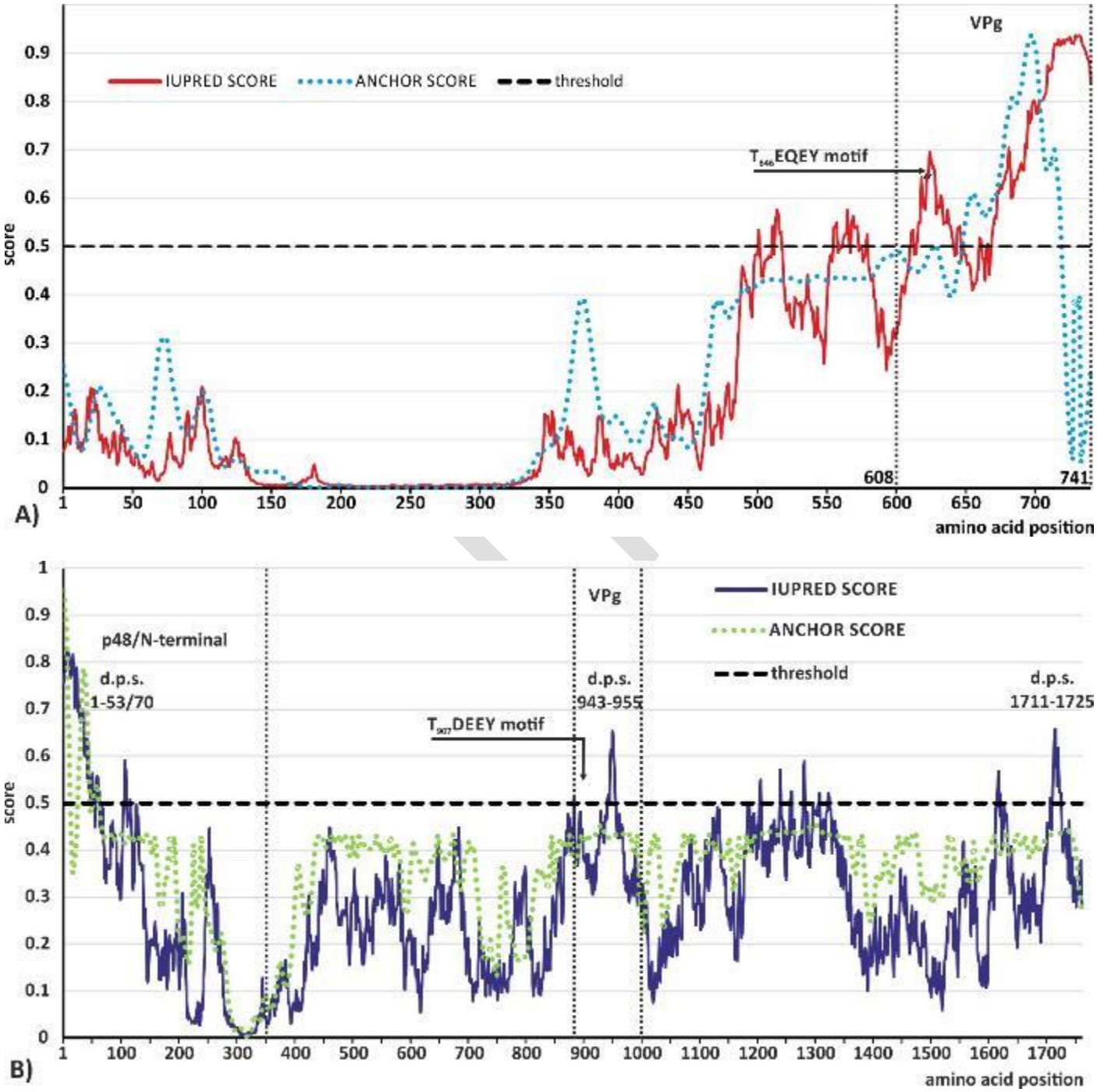
Supplementary – Figure 1.

Bioinformatics result of viral metagenomics study of the randomly selected free-living Norway rat faecal pool (**A**). In this analysis, we focused on the analysis of sequences belonging to the (**B)** astovirus sequences (N=29 reads/contigs) in family *Astroviridae*, to (**C**) hepevirus sequences (N=273 reads/contigs) in family *Hepeviridae*, and to (**D**) norovirus sequences (N=138 reads/contigs) in family *Caliciviridae* belonging to the viruses with RNA containing genome (N=2227). The selected reads/contigs were blasted to GeneBank database by BLAST(n/x), then the reads/contigs were aligned to the closest relative complete sequence. The rodent astrovirus strain HK-1797F (KT946732) in family *Astroviridae*, the *Orthohepevirus C* strain LA-B350 (KM516906) in family *Hepeviridae* and norovirus strain Rn/GV/HKU_KT/HKG/2012 (JX486102) in family *Caliciviridae* viruses were selected as reference to create the genome wide coverage and distribution of the reads/contigs.


A)
N = 2227
Picobirnaviridae (1092)
Picornaviridae (433)
Astroviridae (B)
Caliciviridae (D)
Hepeviridae (C)
Virgaviridae (12)
Secoviridae (17)
Dicistroviridae (25)
other RNA (25)
Totiviridae (43)
Iflaviridae (51)
Nodaviridae (89)
B)
N=29 reads/contigs
sequence coverage (no. of nucleotide identity)
genome length in nucleotide
Ref. genome: KT946732
C)
N=273 reads/contigs
sequence coverage (no. of nucleotide identity)
genome length in nucleotide
Ref. genome: KM516906
D)
N=138 reads/contigs
sequence coverage (no. of nucleotide identity)
genome length in nucleotide
Ref. genome: JX486102

Supplementary – Figure 2.

Prediction of intrinsically unstructured viral protein-genome linked domain in **A**) ORF1a of rat astrovirus and **B**) ORF1 of rat norovirus proteins (https://iupred2a.elte.hu/). The predictions were calculated by IUPred2 structured domains setup. IUPRED: intrinsically unstructured proteins; ANCHOR: protein binding regions in disordered proteins; VPg: viral protein linked to genome; d.p.s.: disordered protein site



Supplementary – Figure 3

The following process represents the quantification of the study strains in the faecal and organ specimens. The nucleic acid concentration (ng/µl) of the PCR products chosen as standards was measured with a nanodrop, and then the concentrations of the reference points (virus copy/µl) were calculated from the measurements. A dilution series was prepared from a reference product of known concentration and weighed on a qPCR machine (Rotor-Gene Q) using Maxima SYBR Green chemistry (ThermoFisher Sci.). Based on the Ct values determined from the automatically selected thresholds, the equations of the dilution series, reaction efficiencies and R^2 values were determined. Substituting the Ct values of the unknown samples into the equation of the dilution series, the virus concentrations in the faecal and organ samples were calculated and reported as copy/µg.

		rat astrovirus strain NR/rat01-AstV/HUN/2017 (MW149253)					**rat norovirus strain NR/rat01-NoV/HUN/2017 (MW174170)**					**rat hepavirus strain NR/rat08-HEV/HUN/2018 (MW149254)**				
NanoDrop measurement (A260/A280)	Standard concentration:	533.02 ng/µl (514.9; 525; 559.2)					433.6 ng/µl (419.6; 552.6; 428.6)					530.75 ng/µl (532.5; 528.3; 535.5; 526.7)				
	NTC* concentration:	495.9 ng/µl (492.1; 501.7; 494)					316.3 ng/µl (242.5; 322.4; 383.9)					508.43 ng/µl (500.1; 509; 516.2)				
	Effective concentration (Δ):	37.13 ng/µl					117.3 ng/µl					22.32 ng/µl				
Oligo Calc*	PCR product size (bp):	449 bp					237 bp					778 bp				
	Molecular weight of the dsDNA template (g/mol):	277330.4 g/mol					146314 g/mol					480652.2 g/mol				
Rotor-Gene Q (Software 2.3.1.49)	Calculated standard concentration (copy/µl):	8.0624×10^{10} copy/µl					4.8278×10^{11} copy/µl					2.7964×10^{10} copy/µl				
	dilution series:	8.0624×10^{8}	8.0624×10^{6}	8.0624×10^{4}	8.0624×10^{3}	8.0624×10^{2}	4.8278×10^{7}	4.8278×10^{5}	4.8278×10^{3}	4.8278×10^{2}	4.8278×10	2.7964×10^{8}	2.7964×10^{6}	2.7964×10^{4}	2.7964×10^{3}	2.7964×10^{2}
	Ct values:	3.15	7	13.84	17.71	21.72	10.38	17.32	24.5	28.43	32.08	2.56	15.55	18.35	17.99	23.98
	Threshold:	0.039					0.4231					0.014				
	Reaction efficiency:	0.87425					0.88779					1.10647				
	R^2:	0.99834					0.99962					0.87356				
	Equation used to calculate the concentration:	10^(-0.273 * Ct + 8.767)					10^(-0.276 * Ct + 10.504)					10^(-0.324 * Ct + 10.122)				

*http://biotools.nubic.northwestern.edu/OligoCalc.html; NTC = no template control

Supplementary – Table 1

Family	Genus	strain(s)	genome region	Reaction type	Primer name/nt position	5'-3' sequence	Product size
Astroviridae	Mamastrovirus	in this study	ORF2	read detection	S24-Astro-4376-R	AGTATCAAACCAGCCCTGTCTCAT	448bp
					S24-Astro-3928-F	TTGCACGCGCGGCTCGAGCAGCT	
			ORF1b	SCR	UNIV-MAstro-R	TTTGG**W**CC**N**CCCC**H**CCAAA	340
					UNIV-MAstro-F	T**N**TTTGG**N**ATGTGGGT**N**AA	
		in this study	ORF2	qPCR	ratAstro-qPCR-4349-R	AGGAATGTCACGCGCTTCAAGAGT	123bp
					ratAstro-qPCR-4226-F	GGTCTCGCTGAACCAGTCTGG	
Caliciviridae	Norovirus	in this study	ORF1	read detection	S24-Noro-4855-R	GGGCCCACGTGTCCAATCAAGCTG	236bp
					S24-Noro-4619-F	GATGATGAGATTGTTTCCACCAAT	
			ORF2	screen	rodentNoro-SCR-R	ACCACCTTGCCAGCAGTAAA	400bp
					rodentNoro-SCR-F	TTTGT**K**AATGAGGATGAGTGA	
			ORF1	qPCR	ratNoro-qPCR-4830-R	CTCTGTATGGAATTCCTGTCCAAT	122bp
					ratNoro-qPCR-4708-F	CGACAAGACTGATGGCCCAATCAC	
Hepeviridae	Orthohepevirus C	in this study	ORF2	read detection	S24-HEV-6431-R	GCTGGCCGGTAGCAACATT	238bp
					S24-HEV-6193-F	TCATGATATAGACTTAGGGCTA	
			ORF1	screen	ratferretHEV-SCR-R	CTGTT**KY**TTGGTCGCATCCG	180bp

					ratferretHEV-SCR-F	TCTCGCCA**R**CGCTGCATATCT	
		in this study	ORF2	qPCR	ratHEV-qPCR-5890-R	GGCTGTGGTACGGAACCG	93bp
					ratHEV-qPCR-5797-F	ATTAGGCCTGCTTGACATTGC	
SCR: screening primer; qPCR: quantitative polymerase chain reaction primer; Base analogues: **W** (A+T); **K** (G/T); **Y** (C/T); **R** (A/G); (**H** (A/C/T); **N** (A/C/G/T)							