

DISCOVERY OF NOVEL VIRUSES AND VIRUS-LIKE AGENTS IN ANIMALS AND HUMANS BY MOLECULAR METHODS AND VIRAL METAGENOMICS

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During the 4 research years total of 21 taxonomically novel virus species and at least 10 novel virus genera were identified, characterized and published in 35 peer-reviewed scientific papers (Σ IF=133.779) and in 22 book chapters. Promising samples were subjected to viral metagenomics and next generation sequencing (MiSeq/HiSeq, Illumina) methods in years 2015 (n=45 specimen pools), 2016 (n=44 specimen pools) and 2018 (n=44 specimen pools). Related to the specimens in 2015, the senior scientist (RG) was participated in a 21-day-long study tour at the Blood Systems Research Institute (San Francisco, USA) financed by OTKA. The complete novel viral genomes were determined by combined molecular biological methods some of them were developed in our laboratory (*Mol Biotech*, 2015;57:974-981). Molecular epidemiological methods were used to investigate the abundance, endemic presence, diversity, evolutionary connections and potential clinical and pathogenetic background of the identified viruses.

Virome and novel viruses related to 7 virus families (See points I-VII below) were investigated and completed in total of 19 host species (in class of mammal, bird, reptile, amphibian, fish, and arthropods) from stool, blood, nasopharyngeal, specific organ and tissue samples.

I. Picornaviridae (+ssRNA): A total of 20 complete genomes of picornaviruses from 14 different hosts were determined. Eight of these picornaviruses are founding members of six novel genera (5 are already accepted officially * by the International Committee of Taxonomy of Viruses, ICTV):

The first two picornaviruses, *Ampivirus** (*J Gen Virol*, 2015;96(9):2607-2613) and “*Livupivirus*” (*Arch Virol*, 2017;162:1043-1050), were discovered in lower vertebrates, in amphibian, in smooth newt (*Lissotriton vulgaris*).

A novel tortoise (*Indotestudo forsteni*) picornavirus, *Rafivirus**, was the first known reptile picornavirus (*Arch Virol* 2015;160(5):1319-1323).

A novel picornavirus, *Harkavirus**, was identified from kestrel (*Falco tinnunculus*) (*Infect Genet Evol* 2015;32:425-431).

A novel picornavirus, *Rabovirus**, was identified from urban rats (*Rattus norvegicus*) (*Arch Virol* 2015;160(10):2569-2575).

The virome of a single diarrhoeic chicken (*Gallus gallus domesticus*) showed that a total of six different avian picornavirus were identifiable which belong to genus *Sicinivirus* (the first complete genome), *Gallivirus* (founded previously by our group), *Tremovirus*, *Avisivirus* (founded previously by our group), *Orivirus** and “*Phacovirus*” (*Virology*, 2016;489:63-74).

A novel picornavirus was detected for the first time from rabbits (*Oryctolagus cuniculus* var. *domestica*) (*Infect Genet Evol* 2016;37:117-122).

A novel genotype of full-length pasiviruses (PaV-A3) from swine (*Sus scrofa domestica*) was determined (*Arch Virol*, 2015;160(5):1363-1366).

A novel megrivirus-related avian picornavirus species (genus *Megrivirus*) was detected at the first time from carnivorous wild bird, western marsh harrier (*Circus aeruginosus*) (*Arch Virol* 2017;162(9):2781-2789).

A novel, rodent-borne Ljungan/Sebokele-like picornavirus species (genus *Parechovirus*) was detected in birds of prey, common kestrel (*Falco tinnunculus*) and red-footed falcon (*F. vespertinus*). Because Ljungan virus is important agent in rodents and possibly in humans (as zoonotic agent) the knowledge of the host species spectrum

(reservoirs) and the genetic diversity (sensitivity of the detection methods) of Ljungan-like viruses is crucial to assess their potential transmission and importance as human pathogens (*Infect Genet Evol* 2017;55:14-19).

A novel dicipivirus species (genus *Dicipivirus*) was detected in wild Northern white-breasted hedgehog (*Erinaceus roumanicus*). This unique picornavirus has dicistronic genome organization (*Arch Virol*, 2018;163:175-181).

A novel picornavirus species was detected from a migratory waterfowl, greater white-fronted goose (*Anser albifrons*). The goose picornavirus genome further increases the diversity of known avian picornaviruses (*Arch Virol*, 2018;163:1087-1090).

A novel passerivirus species (genus *Passerivirus*) was detected in an outbreak of enteritis with high mortality among home-reared estrildid finches (*Uraeginthus* sp.). Beside the intestinal symptom (diarrhoea), the extra-intestinal virus presence suggests a generalized infection in this fatal disease for which the passerivirus is might be a potential causative agent (*Arch Virol*, 2018;163:1063-1071).

A highly divergent picornavirus (“Cyprivirus”) infecting the gut epithelia of experimental animal, zebrafish (*Danio rerio*), was discovered from specimens collected from research institutions world-wide (*Zebrafish*, 2019; *in press*).

Based on our work on the first published outbreak of human salivirus (*Arch Virol* 2016;161:1671-1677) we published an invited review on saliviruses (*Rev Med Virol*, 2017;27(1):e1904).

II. Astroviridae (+ssRNA genome):

A complete genome of a novel astrovirus species was discovered from European rollers (*Coracias garrulus*), which is the first mamastrovirus (mammalian-borne astroviruses) from bird (*Infect Genet Evol* 2015;34:114-121).

III. Parvoviridae (ssDNA genome):

A novel parvovirus species was discovered from swine (*Arch Virol*, 2016;161:1033-1037).

IV. Hepeviridae (+ssRNA genome): Two novel complete genomes of hepevirus species and a novel hepevirus genus were discovered from 3 different hosts:

A divergent hepatitis E virus (HEV)-like orthohepevirus was detected in birds of prey, in common kestrel (*Falco tinnunculus*) and red-footed falcon (*F. vespertinus*) genetically related to mammalian HEVs (*Infect Genet Evol* 2016;43:343-346).

A novel avian-like orthohepevirus was detected in little egret (*Egretta garzetta*) at the first time in wild bird which distantly related to the known avian (chicken) HEVs (*Infect Genet Evol* 2016;46:74-77). Our result suggests that birds may be important reservoir of HEV generally and could be harbor/infected with genetically highly divergent strains of HEV.

A genetically highly diverse and abundant hepevirus-like virus determined from agile frog (*Rana dalmatina*) tadpole (*Infect Genet Evol* 2018;65:112-116).

V. Rhabdoviridae (-ssRNA genome):

A novel rhabdovirus “Riverside virus”, was identified from trapped mosquitoes (*Infect Genet Evol* 2016;39:336-341).

VI. Picobirnaviridae (dsRNA genome):

Novel picobirnaviruses were discovered from chicken (*Gallus gallus domesticus*) and a bacteriophage-nature of picobirnavirus was proposed for the first time based on the discovered functional prokaryotic Shine-Dalgarno genome sequence signature (*Arch Virol*, 2018;163(12):3455-3458; *Virology*, 2018;525:62-72).

VII. Retroviridae (+ssRNA genome):

A novel species of endogenous retrovirus from black Syrian hamster (*Mesocricetus auratus*) with squamous cell skin tumor was discovered. The proviral genome has typical full-length betaretrovirus genome organization with genetic relation to mouse mammary tumor virus (MMTV) (*Viruses*, 2019 submitted).

Based on the preliminary metagenomics data there are further 15-20 potential novel viruses in our hands. Genomic characterizations and publication of these viruses are still in progress.

The pathogenesis and the etiological connections of some of the viral infections were also investigated and successfully confirmed:

1. Co-infection with coxsackievirus A5 (*Picornaviridae*) and norovirus GII.4 (*Caliciviridae*) detected as a possible trigger of the first episode of a severe encephalopathy in a six-year-old child with the intermittent form of maple syrup urine disease (MSUD) (*Arch Virol* 2017;162(6):1757-1763).

2. A neurovirulent/neuroinvasive porcine astrovirus (type 3) was detected in outbreaks of encephalomyelitis, weakness, and paralysis with high mortality rate among weaned pigs (*Emerg Infect Dis*, 2017;23(12):1999-2010). This is a novel neurologic disease entity in previously unknown encephalitis in domestic pigs. The presence of porcine astrovirus type 3 in neurons was confirmed by combination of histology, histopathology, RT-PCR, real-time PCR and ISH in brain and spinal cord, particularly in brains stem, cerebellum (Purkinje cells) and cervical spinal cord. Interestingly, astrovirus was generally undetectable in feces but present in respiratory samples, indicating a possible respiratory infection. This study shows for the first time that astrovirus could cause common, neuroinvasive epidemic disease in pigs. Patent application is submitted to the European Patent Office. In vivo experimental animal study with co-operations a pharmaceutical company is ongoing. A review was published about the novel neurovirulent astroviruses (*Clin Microbiol Rev*, 2018;31:e0040-18).

3. Our international consortium (NoroNet, <http://www.rivm.nl/en/Topics/N/NoroNet>) was raised the attention for the pandemic potential of human norovirus (*Caliciviridae*) GII.17-2014 (Kawasaki) in July, 2015 (*Eurosurveillance*, 2015;20(26):8-15). This virus was first identifiable in Hungary in October, 2015 during a gastroenteritis outbreak in a Danubian cruise ship. Parallel with the increase of the number of norovirus outbreaks, this genotype became the predominant type in Hungary between October, 2015 and January, 2016 (*Epinfo*, 2016;23(2):13-16).

4. The global molecular surveillance of human norovirus in gastroenteritis outbreaks of period between 2005 and 2016, which includes full term data from Hungary published in *The Lancet Infectious Diseases* (2018;18(5):545-553). This molecular epidemiological analysis collected from the NoroNet network from 19 countries in 4 continents shows the long term global trends and major shifts in the genetic composition of the noroviral population circulating in human, based on 16.635 norovirus sequences, in the pre-vaccination area.

5. Connection between the Merkel cell carcinoma (MCC) and the Merkel cell polyomavirus (MC) was investigated in epidemiological, clinic-pathological and virological point of views using tumor specimens from the PTE Clinical Centre (2007-2012). The MC was detected in 3 of the 8 MCC tumors. Based on the presence (or absence) of MC polyomavirus, characteristic histopathological features were identified which showed connection with the clinical prognosis (*Pathol Oncol Res* 2016;22(1):71-77).

6. The clinical significance and molecular epidemiology of severe human parechovirus (*Picornaviridae*) infections (gastroenteritis, sepsis-like/neurological syndromes) in newborns and infants were also investigated for the first time in Hungary (*Orv Hetil*, 2019. 160(10):391-400).

RG is a co-author of the ICTV Virus Taxonomy Profile: *Picornaviridae* (*J Gen Virol*, 2017;98:2421-2422); first author of Porcine astroviruses and co-author of Porcine caliciviruses in *Diseases of Swine (11th edition, John Wiley & Sons, 2019, in press)* and 10 chapters of the *Hungarian Medical Microbiology Textbook (3th edition, 2019, in press)*.

There was no personal change in the staff members (RG, PP, BÁ and HKB). There were total of 6 graduate students (HR, BN, AÁ, CsZs, NO and OK) in the project: 4 of them became an authors of 7 peer-reviewed papers and all of them had presentations in scientific

conferences. HR and CsZs won a first prize and special awards in at the XXXIII. National Student's Research Conference (OTDK, Pécs) in 2017, respectively. In the field on this research RG became the Doctor of Science (DSc, 2015) granted by the Hungarian Academy of Science (MTA). BÁ and PP were granted with the Bolyai scholarship of the MTA. RG is a member of the *Picornaviridae* Study Group and the *Hepeviridae* Study Group of ICTV.

In summary, during the four years the virome of animal species (including class of arthropod, fish, amphibian, reptile, bird and mammal) were investigated using viral metagenomics/next generation sequencing methods and taxonomically novel RNA and DNA viruses were discovered. Novel, genetically divergent viruses were detected in different host species and in certain cases the pathogenesis was also successfully confirmed. We maintained the international co-operation in the fields of viral molecular epidemiology and novel virus discovery with world-class laboratories and experts in virology. We thank NKFIH/OTKA for the trust and the uninterrupted financial supports.

Discovery of novel viruses (summary report):

genus name "suggested name"	taxonomic position (notes)	host species	GenBank acc. number	year of publication	reference
family <i>Picornaviridae</i> (+ssRNA genome)					
"Rafivirus"	novel genus, novel species	turtle (<i>Indotestudo forsteni</i>)	KJ415177	2015	<i>Arch Virol</i> 2015;160:1319-1323
"Harkavirus" previously "Falcovirus"	novel genus, novel species	kestrel (<i>tinnunculus</i>)	KP230449 KP230450	2015	<i>Infect Genet Evol</i> 2015;32:425-431
"Ampivirus"	novel genus, novel species	newt (<i>Lissotriton vulgaris</i>)	KP770140	2015	<i>J Gen Virol</i> 2015;96(9):2607- 2613
"Rabovirus"	novel genus, novel species	rat (<i>Rattus norvegicus</i>)	KP233897	2015	<i>Arch Virol</i> 2015;160:2569-2575
<i>Pasivirus</i>	novel genotype	swine (<i>Sus scrofa domestica</i>)	KM259923	2015	<i>Arch Virol</i> 2015;160:1363-1366
"Phacovirus"	novel genus, novel species	chicken (<i>Gallus gallus domesticus</i>)	KT880670- KT880677	2016	<i>Virology</i> 2016;489:63-74
<i>Sicinivirus</i>	genus		KT880665		
<i>Gallivirus</i>	genus		KT880666		
<i>Tremovirus</i>	genus		KT880668		
<i>Avisivirus</i>	genus		KT880669		
"Orivirus"	novel genus, novel species		KT880667		
<i>Kobuvirus</i>	novel species	rabbit (<i>Oryctolagus cuniculus</i> var. <i>domestica</i>)	KT325852 KT325853	2016	<i>Infect Genet Evol</i> 2016;37:117-122
<i>Salivirus</i>	(the first report of salivirus outbreak, the first complete genome)	human	KT240115- KT240117	2016	<i>Arch Virol</i> 2016;161:1671-1677
"Livupivirus"	novel genus, novel species	newt (<i>Lissotriton vulgaris</i>)	KX463670	2017	<i>Arch Virol</i> 2017;162:1043-1050
<i>Megrivirus</i>	novel species	Western	KY488458	2017	<i>Arch Virol</i>

		Marsh-harrier (<i>Circus aeruginosus</i>)			2017;162:2781-2789
<i>Parechovirus</i>	novel species	kestrel (<i>Falco tinnunculus</i>)	KY645497- KY645499	2017	<i>Infect Genet Evol</i> 2017;55:14-19
<i>Dicipivirus</i>	novel species	hedgehog (<i>Erinaceus roumanicus</i>)	MF188967	2018	<i>Arch Virol</i> 2018;163:175-181
<i>Passerivirus</i>	novel species	finch (<i>Uraeginthus sp.</i>)	MF977321- MF977323	2018	<i>Arch Virol</i> 2018; 163(4):1063-1071.
<i>Kobuvirus</i>	novel species	wild-goose (<i>Anser albifrons</i>)	MF358731	2018	<i>Arch Virol</i> 2018; 163(4):1087-1090.
"Cyprivirus"	novel genus, novel species	zebrafish (<i>Danio rerio</i>)	MH368041	2019	<i>Zebrafish</i> 2019; in press
family Astroviridae (+ssRNA genome)					
<i>Mamastrovirus</i>	novel species	roller (<i>Coracias garrulus</i>)	KT663426 KR057184	2015	<i>Infect Genet Evol</i> 2015;34:114-121
family Parvoviridae (ssDNA genome)					
<i>Protoparvovirus</i>	novel species	swine (<i>Sus scrofa domestica</i>)	KT965075	2016	<i>Arch Virol</i> 2016;161:1033-1037
family Hepeviridae (+ssRNA genome)					
<i>Orthohepevirus</i>	novel species	kestrel (<i>Falco tinnunculus</i>) falcon (<i>F. vespertinus</i>)	KU670940- KU670942	2016	<i>Infect Genet Evol</i> 2016;43:343-346
<i>Orthohepevirus</i>	novel species	little egret (<i>Egretta garzetta</i>)	KX589065	2016	<i>Infect Genet Evol</i> 2016;46:74-77
?	nvel genus?	agile frog (<i>Rana dalmatina</i>)	MH330682	2018	<i>Infect Genet Evol</i> 2018;65:112-116.
family Rhabdoviridae (-ssRNA genome)					
"Riverside virus"	nvel genus	mosquito (<i>Ochlerotatus sp.</i>)	KU248085- KU248087	2016	<i>Infect Genet Evol</i> 2016;39:336-341
family Picobirnaviridae (dsRNA genome)					
<i>Picobirnavirus</i>	novel variants	chicken	MH327933- MH327934	2018	<i>Archives of Virology</i> , 2018;163(12):3455-3458.
<i>Picobirnavirus</i>	novel variants	chicken	MH425579- MH425583, MH425584- MH425590	2018	<i>Virology</i> , 2018;525:62-72.
family Retroviridae (+ssRNA genome)					
<i>Betaretrovirus</i>	novel species	hamster (<i>Mesocricetus auratus</i>)	MK304634	2019	<i>Viruses</i> , 2019; submitted

Publications between 2015 and 2019:

Scientific papers

1. Boros Á, Fenyvesi H, Pankovics P, Bíró H, Phan GT, Delwart E, Reuter G. Secondary structure analysis of swine pasivirus (family *Picornaviridae*) RNA reveals type-IV IRES and parechovirus-like 3'UTR organization
Archives of Virology, 2015. 160(5), 1363-1366.
Impakt faktor: 2,255
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Archives of Virology, 2015.160(5), 1319-1323.
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Infection, Genetics and Evolution, 2015. 34, 114-121.
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5. Reuter G, Boros Á, Tóth Z, Phan TG, Delwart E, Pankovics P. A highly divergent picornavirus in the amphibian, the smooth newt (*Lissotriton vulgaris*)
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Infection, Genetics and Evolution, 2016. 37, 117-122.
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11. Hargitai R, Pankovics P, Kertész AM, Bíró H, Boros Á, Phan GP, Delwart E, Reuter G. Detection and genetic characterization of a novel parvovirus distantly related to human bufavirus in domestic pigs
Archives of Virology, 2016. 161, 1033-1037.
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Impakt faktor: 0
16. Reuter G, Boros Á, Mátics R, Kapusinszky B, Delwart E, Pankovics P. Divergent hepatitis E virus in birds of prey, common kestrel (*Falco tinnunculus*) and red-footed falcon (*F. vespertinus*), Hungary.
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