Arch Virol (2013) 158:2163–2167
DOI 10.1007/s00705-013-1712-9

BRIEF REPORT

Novel seadornavirus (family *Reoviridae*) related to Banna virus in Europe

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Received: 22 February 2013 / Accepted: 29 March 2013 / Published online: 28 April 2013

Abstract Banna virus, whose genome is composed of 12 segments of double-stranded RNA, is a member of the genus *Seadornavirus* in the family *Reoviridae* and is thought to be an emerging mosquito-transmitted human pathogen in Southeast Asia. A novel phylogenetic relative of Banna virus (Balaton virus, BALV, JX947843-JX947850 and KC522611-KC522612) was identified using viral metagenomics in the intestinal contents of freshwater carp (*Cyprinus carpio*) in Hungary. The amino acid sequence identity of Balaton virus to homologous proteins of Banna viruses was 25-26 % for segment 12 (VP12) and 61-62 % for segment 1 (VP1), indicating that Balaton virus potentially represents a novel seadornavirus species. This study demonstrates that seadornaviruses are genetically diverse, not restricted geographically to Southeast Asia and present in an aquatic environment.

Keywords Banna virus · Kadipiro virus · Liao ning virus · Balaton virus · *Reoviridae* · *Seadornavirus* · Double-stranded RNA virus · Europe

Nucleotide sequence data reported here are available in the GenBank database under accession numbers JX947843-JX947850 and KC522611-KC522612.

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Banna virus (BAV), the prototype species of the genus *Seadornavirus* (Southeast Asian dodeca RNA virus) within the family *Reoviridae*, has a genome composed of 12 segments of double-stranded RNA [3]. Banna virus was first isolated from individuals with encephalitis and fever in China (Yunnan Province, Xishuang Banna Prefecture) in 1987 [18]. Banna virus has been also obtained from pigs, cattle and ticks in China [12, 19] and from different species of mosquitoes, which serve as vectors, in Indonesia [5], China [6] and Vietnam [14]. Banna virus is a biosafety level 3 (BSL3) arboviral agent that is pathogenic to humans and thought to be an emerging pathogen or play a part in undiagnosed cases of flu-like symptoms and encephalitis in humans in some areas in tropical and subtropical Asia [2].

The Banna virus genome comprises approximately 21,000 bp, with segment lengths that range between ~3760 bp (segment 1) and 862 bp (segment 12) [3]. Segments 1, 4, 7, 9 and 12 encode the RNA-dependent RNA polymerase (RdRp, VP1), outer-coat protein (VP4), protein kinase (VP7), outer-coat cell attachment protein (VP9) and dsRNA-binding protein (VP12), respectively [3, 8].

Except for a recent mosquito genome analysis in which a DNA copy of genome segment 5 of Liao ning virus was described to possibly be integrated in the genome of *Aedes aegypti* of both Asian and African origin [10, 13], there is no report of these three species of sedornaviruses (Banna virus, Kadipiro virus [KDV] and Liao ning virus [LNV]) outside of Southeast Asia. This study reports a novel Banna-like virus detected by viral metagenomic analysis in the intestinal contents of freshwater carp in Hungary, which extends our knowledge about the genomic diversity and environmental and geographical distribution of seadornaviruses.

In April 2010, intestinal contents were freshly collected from two freshwater carp (*Cyprinus carpio*) during fish

Table 1 Characteristic features and percent amino acid (aa) sequence identity between the novel Banna-like virus (Balaton virus strain Balaton/2010/HUN) and the representative species of the genus *Seadornavirus* based on the available complete amino acid sequences of homologous viral proteins (VP) [1]

Genome segment name (viral protein) of Banna (BAV) virus	Balaton virus (strain Balaton/2010/HUN)						Amino acid sequence identity (%) to homologous viral proteins (VP) of			Putative protein function in seadornaviruses [1]
	GenBank accession no.	Segment length (bp)	Protein length (aa)	5'UTR length (bp)	3'UTR length (bp)	Conserved 5'/3' terminal sequences (5'-3' positive strand)	Banna virus (BAV)	Kadipiro virus (KDV)	Liao ning virus (LNV)	
Segment 1 (VP1)	JX947843	3735	1214	20	70	5'-**GUAAA**AAUUGU…/ …AUAGAACU**GAC**-3'	61-**62** (VP1)	39 (VP1)	38-39 (VP1)	RNA-dependent RNA polymerase
Segment 2 (VP2)	NA	NA	NA	NA	NA	NA	NA (VP2)	NA (VP2)	NA (VP2)	Inner-layer coat protein (T=2 core)
Segment 3 (VP3)	JX947844	2443	727	24	235	5'-**GUAAA**AAAUUU… …AUAGAACU**GAC**-3'	48-**50** (VP3)	34 (VP3)	36 (VP3)	Guanylyltransferase (capping enzyme)
Segment 4 (VP4)	JX947845	2077	629	27	160	5'-**GUAAA**GAAAUU…/ …AGAAGACC**GAC**-3'	47-**48** (VP4)	30 (VP4)	30 (VP4)	Outer coat protein
Segment 5 (VP5)	JX947846	1566	453	32	172	5'-**GUAAA**AAAUUU…/ …AGAAAAACU**GAC**-3'	42-**44** (VP5)	24 (VP6)	25 (VP6)	Non-structural protein
Segment 6 (VP6)	KC522611	1812	437	171	327	5'-**GUAAA**AGGUCU…/ …AAAUGACU**GAC**-3'	38-**39** (VP6)	16 (VP5)	17 (VP5)	Non-structural protein (NTPase)
Segment 7 (VP7)	JX947847	1125	313	39	144	5'-**GUAAA**AAAUUU…/ …AGAAAAAU**GAC**-3'	42-**44** (VP7)	24 (VP7)	21 (VP7)	Protein kinase
Segment 8 (VP8)	KC522612	1122	297	29	199	5'-**GUAAA**AAUUUU…/ …AAAUGACC**GAC**-3'	39-**40** (VP8)	24 (VP9)	27-28 (VP8)	Outer-layer core protein (T=13 core)
Segment 9 (VP9)	JX947848	1105	285	23	224	5'-**GUAAA**AAAUUU…/ …AAAGGACC**GAC**-3'	29-**32** (VP9)	18 (VP11)	14 (VP10)	Outer-coat cell attachment protein
Segment 10 (VP10)	NA	NA	NA	NA	NA	NA	NA (VP10)	NA (VP10)	NA (VP9)	Core protein
Segment 11 (VP11)	JX947850	745	182	74	122	5'-**GUAAA**AAAUUU…/ …GAAAACCU**GAC**-3'	**44** (VP11)	15 (VP12)	17 (VP12)	Non-structural protein
Segment 12 (VP12)	JX947849	842	218	43	142	5'-**GUAAA**AAAAUC…/ …GAAAAACU**GAC**-3'	25-**26** (VP12)	14 (VP8)	9 (VP11)	dsRNA-binding protein

The consensus terminal nucleotides of the Balaton virus sequences and the highest aa sequence identities are indicated by bold letters

NA not available

processing. Carp were caught by line-fishing from the same fishpond, located in Veszprém County, Hungary [4]. The pond is re-stocked regularly (5-6 times per year) with fish from no farther away than 100 km. One sample of intestinal contents diluted in PBS was analyzed directly by enriching for viral-particle-associated nucleic acid using filtration and nuclease treatment followed by sequence-independent random reverse transcription PCR (RT-PCR) amplification and 454 pyrosequencing using 454 GS FLX technology as described previously [9, 16]. The pyrosequencing reads and assembled sequence contigs were compared to the GenBank nucleotide and protein databases using BLASTn and BLASTx, respectively. Specific reverse or forward primers were designed based on the sequence contigs from the pyrosequencing reads to determine the complete viral nucleotide sequences, including the 5' and 3' ends, of the RNA segments using RT-PCR [4] and adapter ligation methods [17]. Degenerate primers were also designed based on the conserved nucleotide sequences (encoding conserved amino acid motifs) of the available seadornaviruses (Banna virus, Kadipiro virus and Liao ning virus) for RT-PCR identification of the missing genome segments of the novel seadornavirus. The primers BALV-VP1-forward (5'-AACGATGAAGCGCCGATGT-3') and BALV-VP1-reverse (5'-TCCTTAGTCCCGTTGAATTG-3') were designed based on the novel seadornavirus genome segment 1, yielding an amplicon of 659 bp length, for RT-PCR screening. PCR-products were sequenced directly using a BigDye Reaction Kit (Applied Biosystems, Warrington, UK) using the PCR primers and run on an automated sequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems, Stafford, Texas, USA). Sequences from representative members of the genus *Seadornavirus* were obtained from GenBank, the study sequences were aligned using Clustal X, and similarity calculations were performed using GeneDoc. Phylogenetic trees of the amino acid sequence alignments were created using the neighbor-joining method based on the Jones-Taylor-Thornton matrix-based model in MEGA5 [15]. Bootstrap values (based on 1000 replicates) for each node are given if >50 %. The GenBank accession numbers of these sequences are JX947843-JX947850 and KC522611-KC522612 (Table 1).

The double-stranded, segmented RNA genome, which was extracted directly from the intestinal contents using the TRIzol method, was resolved in a 0.75-mm thick, 30 % (w/v) polyacrylamide slab gel with a 4 % (w/v) stacking gel using Laemmli's discontinuous buffer system without SDS [11]. Electrophoresis (PAGE) was conducted at 4 °C at a current of 6 mA for 16 and 26 hours, and subsequently, the gel was stained with silver nitrate [7].

A total of 13 sequence reads were initially identified by pyrosequencing, which were then assembled into 11 contigs covering partial (131-451 nucleotide-long) coding regions of 8 of the 12 genome segments of a segmented dsRNA virus with 25 % (VP9) to 77 % (VP1) amino acid

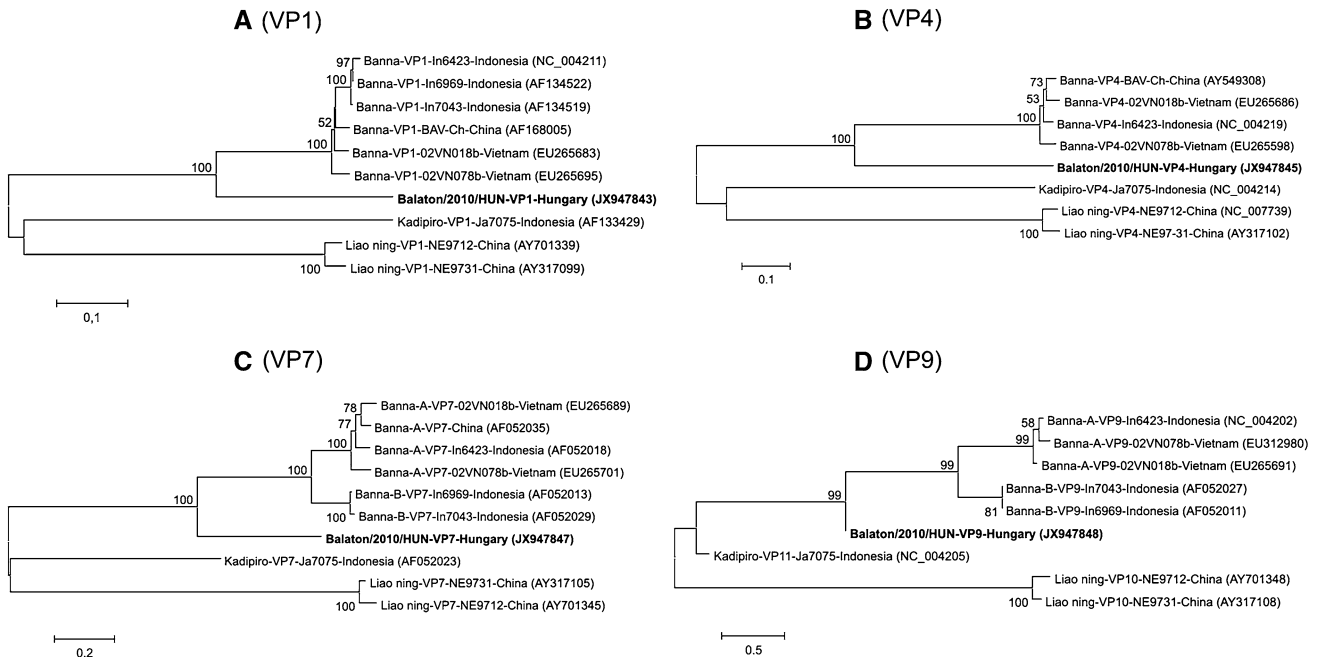

Fig. 1 Phylogenetic analysis of complete amino acid sequences of homologous viral proteins (VP, see Table 1) of Balaton virus (strain Balaton/2010/HUN) using representative members of the species *Banna virus*, *Kadipiro virus* and *Liao ning virus* in the genus *Seadornavirus*: A RNA-dependent RNA polymerase (VP1); B outer-coat protein (VP4); C protein kinase (VP7); D outer-coat attachment protein (VP9) of Banna virus

sequence identity to the corresponding Banna virus sequences. The complete nt (and aa) sequences, including the conserved terminal base pairs of the eight genome segments encoding VP1, VP3, VP4, VP5, VP7, VP9, VP11 and VP12 of this virus (provisionally named Balaton virus; strain Balaton/2010/Hungary), were determined, with segment lengths ranging from 3735 bp (VP1) to 745 bp (VP12) (Table 1). Additional efforts to identify and characterize the missing segments using degenerate primers and adapter ligation methods were successful in two of the four segments: segment 6 (VP6) and segment 8 (VP8) (Table 1). The aa sequence identity of Balaton virus to homologous proteins of Banna virus isolates was 25-26 % for VP12 and 61-62 % for VP1 (Table 1). Much lower levels of aa sequence identity were detected between homologous proteins of Balaton/2010/HUN and members of the other two species of seadornaviruses, Kadipiro virus (14 % to 39 %) and Liao ning virus (9 % to 39 %) (Table 1). In each case, the highest levels of aa sequence identity were detected in VP1 (RdRp polymerase, encoded by segment 1). At the extreme termini of the untranslated regions, conserved nucleotide sequence motifs (GUAAA at the 5' end and GAC at the 3' end of the positive strands) were identified in all of the genome segments characterized (Table 1). Phylogenetic analysis based on the homologous complete aa sequences of VP1, VP4, VP7 and VP9 of Banna virus shows that Balaton virus is a member of the genus *Seadornaviruses* and is most closely related to, but distinct from, Banna virus (Fig. 1) and not related to known fish reoviruses, including aquareoviruses. RNA-PAGE analysis of fish intestinal contents confirmed the presence of a virus with 12 genome segments with migration pattern of 7:3:2 in the sample (Fig. 2).

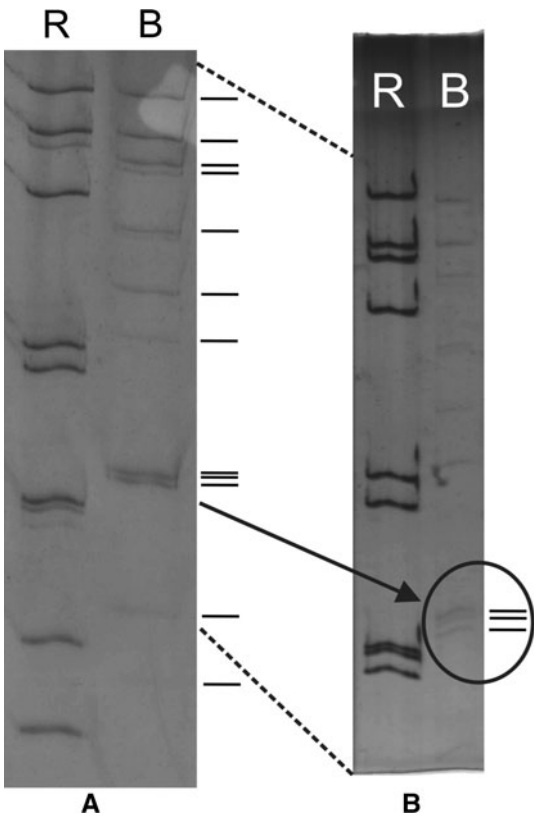

Fig. 2 **a** Side-by-side electrophoretic migration pattern of the 12 double-stranded RNA segments of Balaton virus (strain Balaton/2010/HUN) (lane B) from intestinal contents of freshwater carp and the 11 double-stranded RNA genome segments of human A-type rotavirus (lane R) as a reference in a 30 % polyacrylamide gel (PAGE) stained with silver nitrate. A simplified segment migration profile of the Balaton/2010/HUN genome is visible at the right side. **b** Extended electrophoresis for separating the three nearly equally long genome segments 7, 8 and 9 (circle) of Balaton virus (lane B). Lane R, the same human A-type rotavirus reference shown in panel a

Balaton virus was also identified by RT-PCR, using the screening primers (BALV-VP1-R/BALV-VP1-F) and the RNA-PAGE method, with an identical migration pattern (data not shown), in the intestinal contents of the second freshwater carp that was caught from the same fishpond at the same time. The 659-nucleotide-long, partial viral genome of segment 1 (VP1) was 100 % identical in the two specimens.

Seadornaviruses are arboviruses that are transmitted by different species of mosquitoes in Asia, and Banna virus is thought to be an emerging pathogen in humans [3]. This study reports the detection of a novel seadornavirus related to Banna virus in Europe, identified in the intestinal contents of freshwater carp using viral metagenomic analysis. Inferring the host (and origin) of genetically distinct viruses is problematic, especially if they are found in feces. Feces are known to contain viruses that infect host cells and/or bacteriophages, as well as viruses of dietary origin from consumed plants, insects, and animals [4]. Because freshwater carp are omnivorous, and because mosquitoes (including their larvae developing in a fresh water habitat) are consumed by these fish, in this context and with our present knowledge, we could not exclude the possibility that this novel seadornavirus originated from mosquitoes that were eaten. If this hypothesis is true, a species of mosquito that is present or endemic in Central Europe may therefore be a vector for this novel Banna-like virus. This study also indicates that seadornaviruses are not restricted geographically to Southeast Asia and are present in aquatic environments where they may circulate through aquatic animals such as fish. Whether they retain infectivity after passage through the fish gut or actually infect fish (intestinal) cells will require the isolation of replication-competent virus and inoculation studies. Knowledge of genetically diverse Banna-like virus genome sequences will also facilitate investigations of its potential etiological role in human diseases, including in fever and encephalitis

of unknown origin inside and outside of Asia, using more-sensitive detection methods.

Banna virus (BAV) from China, Vietnam and Indonesia; Kadipiro virus (KDV) from China and Indonesia; and Liao ning virus (LNV) from China are classified as belonging to three distinct species in genus *Seadornavirus*, family *Reoviridae*, based on the current ICTV taxonomy [1, 3]. From full-genome sequence analyses of different Banna viruses, two distinct genotypes have been identified (based on segments 7 and 9) that correlate with virus serotype. Genotype A includes isolates BAV-Ch (China) and BAV-In6423 (Indonesia), while genotype B includes BAV-In6969 and BAV-In7043 (Indonesia) [3]. The proteins translated from segments 7 and 9 show 72 % and 54 % aa sequence identity between genotypes A and B, respectively, while all of the other segments appear to be more conserved, showing 83 % to 98 % identity [3]. Much lower levels of aa sequence identity were detected between homologous proteins of members of different species: 24 % to 42 % between Banna virus and Kadipiro virus, 18 % to 41 % between Banna virus and Liao ning virus, and 21 % to 42 % between Liao ning virus and Kadipiro virus. In each case, the highest levels of aa sequence identity were detected in the viral RdRp polymerase (VP1, encoded in segment 1) [3]. Balaton virus is most closely related to Banna virus, but according to the current ICTV criteria, it is sufficiently distinct to be proposed as a member of a novel species ("Balaton virus"; BALV) in the genus *Seadornavirus*. After this serendipitous observation, further studies will be required to determine the distribution, diversity, pathogenic role, and potential insect and vertebrate vectors and hosts of this novel seadornavirus.

Acknowledgements This work was supported by a grant from the Hungarian Scientific Research Fund (OTKA, K83013). G.R. was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences; and E.D., by NHLBI grant R01-HL105770. We thank Gertrud Domonkos (ÁNTSZ, Pécs, Hungary) for her technical help with PAGE.

Conflict of interest The authors declare that they have no conflict of interest.

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