Short Communication

Genetic characterization of a novel picornavirus detected in Miniopterus schreibersii bats

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Bats are important reservoirs of many viruses with zoonotic potential worldwide, including Europe. Among bat viruses, members of the Picornaviridae family remain a neglected group. We performed viral metagenomic analyses on Miniopterus schreibersii bat faecal samples, collected in Hungary in 2013. In the present study we report the first molecular data and genomic characterization of a novel picornavirus from the bat species M. schreibersii in Europe. Based on phylogenetic analyses, the novel bat picornaviruses unambiguously belong to the Mischivirus genus and were highly divergent from other bat-derived picornaviruses of the Sapelovirus genus. Although the Hungarian viruses were most closely related to Mischivirus A, they formed a separate monophyletic branch within the genus.

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Bats are important reservoirs of many viruses with zoonotic potential (e.g. lyssaviruses, filoviruses, coronaviruses and orthoreoviruses) worldwide, including Europe (Kohl & Kurth, 2014). They are distributed globally, with over 1250 species (Teeling et al., 2005). In addition, several species have effective urbanization strategies and live in close contact with human habitats. This behaviour is becoming frequent due to disturbance of natural roosting locations, such as caves and mines (Uhrin et al., 2012). Among bat viruses, members of the Picornaviridae family remain a neglected group. Although the zoonotic potential of several picornaviruses (PV) had been described, only two studies have reported complete genomic sequence and characterization of a novel picornavirus from the bat species M. schreibersii in Europe. Based on phylogenetic analyses, the novel bat picornaviruses unambiguously belong to the Mischivirus genus and were highly divergent from other bat-derived picornaviruses of the Sapelovirus genus. Although the Hungarian viruses were most closely related to Mischivirus A, they formed a separate monophyletic branch within the genus.

PVs are small, enveloped viruses with a single-stranded, positive-sense RNA genome. The family Picornaviridae belongs to the order Picornavirales and currently consists of 46 species grouped into 26 genera (Knowles et al., 2012; Adams et al., 2013; ICTV Master Species List, 2013). The PV genome encodes a polypeptide, VPg, covalently attached to the 5' end of the viral RNA genome, followed by the 5' UTR region with important internal ribosomal entry site (IRES) elements. The open reading frame, which usually encodes a single polyprotein that is proteolytically cleaved by viral-encoded proteases, is followed by a short 3' UTR and terminated by a 3' poly-A tail.

Recently, we have initiated a survey to describe bat-borne viruses in the European bat fauna, with particular emphasis on RNA viruses, to uncover the occurrence of viruses posing a zoonotic threat in Hungary. In addition, we

The GenBank/EMBL/DDBJ accession numbers for the BatPV sequences were KP054273–KP054278.
exploited the methodology of viral metagenomics, which has been found to be useful for describing new virus diversity in American, African and Asian bats, as well as in bats of West-European countries. As a pilot study, we performed viral metagenomic analyses on Miniopterus schreibersii bat faecal samples. Among bats, *M. schreibersii* is one of the most widespread species in the world, living in large colonies. Schreiber’s bats are distributed in distinct lineages throughout Oceania, Africa, Southern Europe and South-East Asia (Appleton *et al.*, 2004). This study reports the detection of novel mischiviruses, members of a candidate new genus of PVs.

Thirteen faecal samples were collected from a single *M. schreibersii* bat colony, at a cave in Szár somlyó mountain, Hungary (GPS coordinates 45°51′17″ N; 18°24′40″ E), in 2013. In the sampling year the bats had an estimated nursery colony size of 1000 individuals, and 1500 individuals for transient colony size. All captured bats were identified for species by an experienced chiropterologist. The animals appeared to be healthy; there were not any visible physiological or clinical manifestations (i.e. unusual behaviour, lack of active movement, lethargy). Faecal samples were taken from bats that were captured primarily for bat-bandung activities in Hungary. Bats were trapped in 2013 by mist-nets or harp-traps at swarming sites and in their natural foraging habitats. The animals were freed from nets immediately and put into sterile, disposable, highly perforated paper bags individually and were left hanging for a maximum of 10 min, in order to let them defecate. After collecting faecal samples from the bags, bats were released at the netting site. Duplicate sampling was prevented by marking captured bats with nail polish. All samples were collected in 500 μl RNALater RNA Stabilization Reagent (Qiagen) and kept on dry ice until laboratory processing. All bat species in Europe are strictly protected under the Flora, Fauna, Habitat Guidelines of the European Union (92/43/EEC) and the Agreement on the Conservation of Populations of European Bats (http://www.eurobats.org).

Examined bats were handled according the guidelines of Sikes & Gannon (2011). No animals were harmed or invasively sampled during this study. Animal handling processes were conducted by a trained chiropterologist with an appropriate licence for safe handling of bats. This study was approved by The Hungarian National Inspectorate for Environment, Nature and Water (14/2138–7/2011).

After homogenization, stool samples were centrifuged at 17 000 g for 10 min at room temperature. RNA was extracted from 200 μl of supernatant using DiaExtract Total RNA Isolation kit (Diagon) following the manufacturer’s instructions. Samples were subjected to random primed PCR and semiconductor sequencing using the Ion Torrent PGM platform. Library preparation, size selection of library DNA, emulsion PCR, templated Ion Sphere bead enrichment and sequencing was carried out as described in detail elsewhere (Feher *et al.*, 2014). Bioinformatic analysis consisted of the mapping of reads longer than 40 bases against ~1.7 million viral sequences downloaded from GenBank using moderately rigorous mapping parameters (length fraction, 0.6; similarity fraction, 0.8). The CLC Genomics Workbench (http://www.clcbio.com/) was used for de novo sequence assembly and reference mapping of the Ion Torrent reads. The 5’ and 3’ UTR was amplified by 2nd Generation 5′/3′ RACE kit (Roche) using the manufacturer’s recommendations. The reverse transcription-polymerase chain reaction (RT-PCR) amplicons were obtained by using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen), according to the manufacturer’s instructions. Sequences were obtained using ABI PRISM 310 Genetic Analyzer automated sequencer, then edited and aligned using GeneDoc 3.2 and CLUSTAL_X 2.0 software, respectively. The phylogenetic tree was reconstructed with MEGA v5.0 software using the maximum-likelihood method, based on the general time reversible model (GTR + G + I). The number of bootstraps for simulations was 1000. The RNA secondary structure of the 5′-IRES and 3′ UTR region was predicted using Mfold

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**Fig. 1.** Schematic representation of the novel Hungarian BatPV genome organization, including 5′ UTR, 3′ UTR, P1, P2 and P3 regions. Putative cleavage sites and conserved motifs are indicated.
Fig. 2. (a) Predicted secondary structure for 5' UTR of the novel BatPV strain (BatPV-V13/13/Hun). Stem–loops are labelled according to Kapoor et al. (2008) and Duke et al. (1992). Bases that are identical or highly conserved between BatPV, EMCV and CoSV are indicated by grey shading. Initial codon is underlined. Py, polypyrimidine tract. (b) Predicted secondary structure for 3' UTR of the novel BatPV. Grey boxes indicate the conserved nucleotide motif identified previously in EMCV (Duque & Palmenberg, 2001).
In two samples, BatPV sequences were obtained by metagenomic analyses with 416 bp and 465 bp consensus length represented by 14 and 15 sequence reads, respectively. Another four stool samples collected from animals in the same colony were found to contain BatPV RNA when tested by specific primer sets. The genomic RNA sequences identified by viral metagenomics were used to design virus-specific primers which permitted the primer-walking sequencing and the 5'-3' RACE. As a result, the near complete viral genome (7947 nt) was sequenced for six BatPVs. Using the reference BatPV strain (JQ814851), we estimate that approximately 500 nt of sequence data were missing at the 5' UTR. However, the complete coding region was determined and analysed in detail. We identified a single ORF that encoded a large polyprotein of 2284 aa (Fig. 1). The alignment analysis of the new BatPV and other known PVs showed the characteristic genome organization of 5' UTR-L-P1(VP0, VP3, VP1)-P2(2A, 2B, 2C)-P3(3A, 3B, VPg, 3Cpro, 3Dpoly)3' UTR-poly(A). The amino acid lengths of different genome regions were: VP0/VP3 to VP4 and VP2 proteins. The hypothetical cleavage sites within P1, P2 and P3 polyproteins were: VP0/VP3 to VP4 and VP2 proteins. The hypothetical cleavage sites between L-P1-P2-P3 regions were similar (E/D/C) motifs. Also, the catalytic triad (H-E/D-C) of the 3Cpro of BatPV contained H-D-C. It also contained the conserved active protease site GxCG (G1775YCG) with a GxH (G1779DEIR, G2109GLPSG, Y2151GDD and F2200LKR) motifs.

Based upon sequence data and secondary structural similarities, the Hungarian BatPV was predicted to possess a type II IRES (Fig. 2a) similar to those of foot-and-mouth disease virus (FMDV), encephalomyocarditis virus (EMCV) and cosaviruses (CoSV). Interestingly, bat-transmitted picornaviruses that were classified into the Sapelovirus genus possess a type IV IRES (Lau et al., 2011). The main conserved elements, H, I, J, K and L, of the type II IRES were identified in the 5' UTR. Particularly, the predicted secondary structure included a series of signature elements characteristic of type II IRES, e.g. the GNRA tetranucleotide and two A/C-rich loop regions at the apical parts of the I domain, as well as the A-rich loop at the junction of the J and K domains. Domain F within the 5' UTR of BatPV contained a sequence of 13 nt (AGGGGUUCUAUCCU), identical to that found in CoSV, while domain H contained 9 nts (GGUCUUUCC), identical to EMCV. There were also high nucleotide similarities in the case of domains D and J between BatPV, CoSV, FMDV and EMCV. Additionally, BatPV also contained an 8 nt oligopyrimidine tract immediately downstream of the last stem–loop, L (Kapoor et al., 2008; Duke et al., 1992). Interestingly, the region from positions 329 to 440 showed both sequence and structural similarities to that of CoSV, consisting of putative stem–loops D, F and G, and a 20 nt polypyrimidine tract (positions 364–383). The 3' UTR (Fig. 2b) showed 76% identity to the prototype Mischivirus A (Wu et al., 2012) and was determined to be 211 nt (including the stop codon). In addition, the 3' UTR shared 88% identity to Theiler’s murine encephalomyelitis viruses (TMEVs) (Buckwalter et al., 2011; Law & Brown, 1990; Liang et al., 2008; Myoug et al., 2007; Peever et al., 1987; Sorgeloos et al., 2013) between nt 71 and nt 110. Using Mfold (Zuker, 2003), the secondary structure of the new BatPV 3' UTR was predicted to consist of six domains (A–F). Domain C contained the loop AAGCCA88–93 motif present in domain I of EMCV, while domain D contained a variant sequence (CUUCUCUU) that resembled the UCUUCU motif of EMCV (Duque & Palmenberg, 2001). The 3' UTRs of the known picornaviruses contain stem–loops of variable size and number, which may be specific binding sites of viral or cellular proteins (Rohll et al., 1995). The complexity of the Hungarian BatPV 3' UTR suggested the same role in virus replication; however, this hypothesis remains elusive until a cell-culture system for BatPVs becomes available.

Full coding sequences of BatPVs have only been described from China, representing the Sapelovirus and Mischivirus genera (Lau et al., 2011; Wu et al., 2012). Phylogenetic analyses (Fig. 3) of the entire coding region showed that the new BatPVs belong to the Mischivirus genus and they are most closely related to Mischivirus A, the only described member of the genus detected in *M. schreibersii* bats from China (Wu et al., 2012). The complete P1 (2429 nt, 810 aa), P2 (1727 nt, 576 aa) and P3 (2402 nt, 801 aa) regions show 32–37%, 35–39% and 29–32% identity with the prototype reference strain identified previously by Wu et al. (2012). Phylogenetic relationships were inferred using the neighbour-joining method (Hillis et al., 1996; Felsenstein, 1985) implemented in MEGA5 (Tamura et al., 2011) and the Phylip package (Felsenstein, 2005). Phylogenetic trees were visualized using Treeview (Page, 1996). From the alignment analysis, there was no evidence of proteolytic cleavage of the putative VP0 to VP4 and VP2 proteins. The hypothetical cleavage sites within P1, P2 and P3 polyproteins were: VP0/VP3 (GLSNQ122/APPV), VP3/VP1 (PMLNE654/GTN2), 2A/2B (EEENPG963/PDSL), 2B/2C (LKIDEA1575/GENV), 3A/3B (PAQTQ1289/GAYD), 3B/3C (KLQLQ1600/GPGT) and 3C/3D (KLAEDE1847/GFLT). Interestingly, the hypothetical cleavage sites between L-P1-P2-P3 regions were similar (E/D/G) as follows: L/P1 (DLKDE97/GGNS), P1/P2 (PRTTE907/GPPP) and P2/P3 (NLVFE1498/GPND). With the exception of cleavage sites VP0/VP3 and VP3/VP1, the same cleavage pattern was observed between the Hungarian BatPV strains and the prototype reference strain identified previously by Wu et al. (2012). Using the conserved domain database (CDD) search (Marchler-Bauer et al., 2011), RhV-like capsid domains (cd00205), 2C RNA helicase (pfam00910), 3C cysteine protease (pfam00548) and 3D RdRp (cd01699) characteristic picornaviral motifs were recognized. The VP0 displays a GxxNxx (GxGxGNS) motif for potential myristylation. Protein 2A possessed a conserved cleavage site DXExPG/P (DxExPG), while similarly to other picornaviruses, protein 2C contained the conserved NTP-binding GxxGxGKS (G1282KPGTGKS) and helicase DDLxQ (D1332DGLQ) motifs. Also, the catalytic triad (H-E-D-C) of the 3Cpro of BatPV contained H-D-C. It also contained the conserved active protease site GxCG (G1775YCG) with a GxH (G1779DEIR, G2109GLPSG, Y2151GDD and F2200LKR) motifs.
Fig. 3. Phylogenetic analyses of the novel BatPV strains detected in Hungary. Phylogenetic trees were reconstructed with MEGA v5.0 software using a neighbour-joining algorithm with Maximum Composite Likelihood parameter model based on nucleic acid sequences of a 6852 bp coding region of the genome. Bat-derived picornaviruses, detected previously by others, are indicated in grey. Hungarian BatPV strains identified in this study are marked in bold face.
amino acid identity to other bat-derived picornaviruses from the Sapelovirus genus. On the other hand, amino acid identities between the Hungarian BatPVs and Mischivirus A were 73%, 73–74% and 67%, respectively. The main amino acid motifs found in the reference Mischivirus A species have also been described in our BatPV strains. When compared to the reference Mischivirus A species, minor differences were found in the length of the L protein (6 aa shorter), the P2 polyprotein (1 aa longer) and the P3 polyprotein (3 aa shorter).

In the present study, we report the first molecular data and genomic characterization of a novel picornavirus from the bat species, M. schreibersii in Europe. Based on the phylogenetic analyses, the novel BatPVs (BatPVs) unambiguously belonged to the Mischivirus genus and was highly divergent from other BatPVs of the Sapelovirus genus. Although the Hungarian viruses were most closely related to Mischivirus A, they formed a separate monophyletic branch within the genus. Genome organization of the new BatPVs (BatPVs), along with the hypothetical cleavage sites and characteristic amino acid motifs were also similar to the prototype Mischivirus A. According to the current guidelines of the International Committee on Taxonomy of Viruses (ICTV) (http://www.picornastudygroup.com/definitions/genus_definition.htm), members of a genus should normally share phylogenetically related P1, P2 and P3 genome regions, each sharing >40 %, >40 % and >50 % amino acid identity, respectively. It remains to be clarified whether the occurrence of mischiviruses follows a particular geographical pattern and can be linked to different lineages of the widespread M. schreibersii bat species suggestive of co-evolution and/or co-adaptation.

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