ORIGINAL ARTICLE

Complete nucleotide and amino acid sequences and genetic organization of porcine kobuvirus, a member of a new species in the genus *Kobuvirus*, family *Picornaviridae*

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Abstract *Kobuvirus* is a new genus in the family *Picornaviridae*. Two species are currently known: *Aichi virus* (human kobuvirus) and *Bovine kobuvirus* (U-1). In this study, the complete nucleotide and amino acid sequences and genetic organization of porcine kobuvirus (Kobuvirus/swine/S-1-HUN/2007/Hungary, EU787450) were determined. The structure of the S-1-HUN genome, VPg–5′UTR–leader protein–structural proteins (VP0, VP3, VP1)–non-structural proteins (2A–2C, 3A–3D)–3′UTR–poly(A) tail, was found to be typical of picornavirus. The 8210-nucleotide (nt)-long RNA genome contains a large open reading frame (7467 nt) encoding a potential polyprotein precursor of 2488 amino acids (aa) that has 57/56% and 63/64% nt/aa identity with Aichi virus and U-1, respectively. The 5′UTR contains a hepacivirus/pestivirus-like internal ribosomal entry site (IRES type IV group-B-like) with conserved pseudoknot, II and IIIa–f domains. A tandem repeat (a 30-amino-acid-long motif) was detected in 2B. Thirty-nine (65%) of the 60 fecal samples from pigs under the age of 6 months at the tested farm were positive (the incidence was 90% under the age of 3 weeks). Porcine kobuvirus belongs to a potential new species—the third—in the genus *Kobuvirus*.

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Introduction

Picornaviruses (family *Picornaviridae*) are small, non-enveloped viruses with single-stranded, positive-sense genomic RNA and are divided into eight genera: *Enterovirus*, *Aphthovirus*, *Cardiovirus*, *Hepatovirus*, *Parechovirus*, *Erbovirus*, *Teschovirus* and *Kobuvirus* [8, 23]. Most picornavirus genera consist of two or more species [18]. To date, kobuviruses are comprised of two species: *Aichi virus* and *Bovine kobuvirus*, each possessing a single serotype. Aichi virus (strain A846/88) was isolated from a stool sample from a human with gastroenteritis in 1991 [25]. Bovine kobuvirus (strain U-1) was detected in bovine sera and also in feces samples from clinically healthy cattle in 2003 [28]. Both human and bovine kobuviruses were first isolated in Japan. Recently, kobuviruses have also been detected in humans in other countries in Asia [17], Europe [1, 15] and South America [15] and in calves with diarrhea in Southeast Asia [12].

The Aichi virus and the bovine kobuvirus genomes are 8280 and 8374 nucleotide (nt) long, respectively, and both encode a single polyprotein (2432 aa and 2463 aa). This polyprotein has a clear similarity with other picornaviruses. However, some picornaviruses have unique characteristics, such as the genome organization of kobuviruses. Kobuviruses have a leader (L) protein and only three structural proteins, VP0, VP3 and VP1 [26]. The kobuvirus 2A protein contains conserved H-box/NC motifs that are characteristic of a family of cellular proteins involved in the control of cell proliferation [7]. The genetic identity between Aichi and U-1 viruses is between 47.7% (3′UTR) and 70.8% (3D region).

In this study, a complete nucleotide sequence and a detailed genomic organization of a new kobuvirus (Kobuvirus/swine/S-1-HUN/2007/Hungary), which was serendipitously detected by RT-PCR in Hungary in fecal

samples from healthy domestic pigs [20], are presented. Generic kobuvirus screening primers were also designed and used for an epidemiological study.
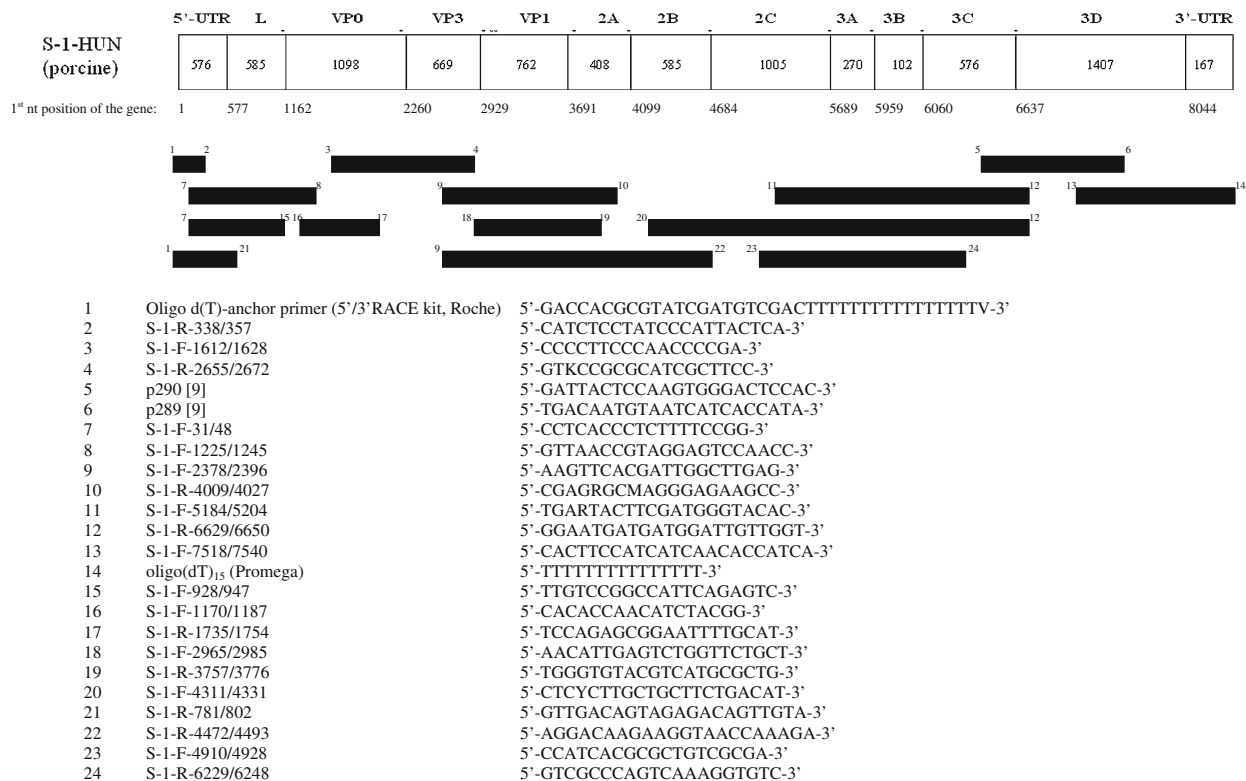
Materials and methods

Specimens

Fecal samples were collected from 60 healthy piglets (*Sus scrofa domestica*) in four age groups (15 specimens per group under the age of 10 days, 3 weeks, 3 months and 6 months) from a farm located in eastern Hungary in February 2007. The aim of the study was to detect porcine calicivirus (norovirus and sapovirus) in domestic pigs by reverse transcription-polymerase chain reaction (RT-PCR) using the generic primer-pairs p289/p290 designed for the calicivirus RNA-dependent RNA polymerase gene (319 nt for norovirus or 331 nt for sapovirus) [9, 20].

RNA isolation and RT-PCR

RNA isolation and RT-PCR were performed as described previously [19]. Briefly, nucleic acid was extracted from 300-μl stool suspensions using the TRIzol Reagent (Invitrogen). The RT reaction was performed in 50 μl of reaction mixture containing 10× PCR buffer (Sigma-Aldrich), dNTP (10 mM), negative-sense primer, rRNasin (40 U/μl), M-MLV-RT (200 U/μl), nuclease-free water and 3 μl of the extracted RNA for 1 h at 42°C. For PCR, 50 μl of 1× PCR buffer containing positive-sense primer and Taq polymerase (Dupl-A-Taq DNA Polymerase, ZenonBio, Szeged, Hungary) was added. The thermocycle program included 3 min at 94°C, 40 cycles for 30 s at 94°C, 90 s at 49°C (or 37°C) and 60 s at 72°C, and a final 10-min extension at 72°C, in a PTC-100™ (MJ Research, Inc., Watertown, Massachusetts). For the determination of the complete nucleotide sequence of porcine kobuvirus strain S-1-HUN, sequence-specific oligonucleotides were designed on the basis of the conserved regions of Aichi (AB040749) and bovine U-1 (AB084788) kobuviruses and the actually available overlapping S-1-HUN nucleotide sequences (Fig. 1). The extreme 5′ and 3′ ends of the genome were determined using a 5′/3′RACE kit (2nd generation, Roche) and oligo(dT)$_{15}$ (Promega), respectively (Fig. 1). Generic kobuvirus primers were selected based on the conserved sequences of the Aichi virus (human), U-1 (bovine) and S-1-HUN (swine): UNIV-kobu-F (forward, 5′-TGGAYTACAAG(/R)TGTTTTGA

#	Primer	Sequence
1	Oligo d(T)-anchor primer (5'/3'RACE kit, Roche)	5'-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTTTV-3'
2	S-1-R-338/357	5'-CATCTCCTATCCCATTACTCA-3'
3	S-1-F-1612/1628	5'-CCCCTTCCCAACCCCGA-3'
4	S-1-R-2655/2672	5'-GTKCCGCGCATCGCTTCC-3'
5	p290 [9]	5'-GATTACTCCAAGTGGGACTCCAC-3'
6	p289 [9]	5'-TGACAATGTAATCATCACCATA-3'
7	S-1-F-31/48	5'-CCTCACCCTCTTTTCCGG-3'
8	S-1-F-1225/1245	5'-GTTAACCGTAGGAGTCCAACC-3'
9	S-1-F-2378/2396	5'-AAGTTCACGATTGGCTTGAG-3'
10	S-1-R-4009/4027	5'-CGAGRGCMAGGGAGAAGCC-3'
11	S-1-F-5184/5204	5'-TGARTACTTCGATGGGTACAC-3'
12	S-1-R-6629/6650	5'-GGAATGATGATGGATTGTTGGT-3'
13	S-1-F-7518/7540	5'-CACTTCCATCATCAACACCATCA-3'
14	oligo(dT)$_{15}$ (Promega)	5'-TTTTTTTTTTTTTTTT-3'
15	S-1-F-928/947	5'-TTGTCCGGCCATTCAGAGTC-3'
16	S-1-F-1170/1187	5'-CACACCAACATCTACGG-3'
17	S-1-R-1735/1754	5'-TCCAGAGCGGAATTTTGCAT-3'
18	S-1-F-2965/2985	5'-AACATTGAGTCTGGTTCTGCT-3'
19	S-1-R-3757/3776	5'-TGGGTGTACGTCATGCGCTG-3'
20	S-1-F-4311/4331	5'-CTCYCTTGCTGCTTCTGACAT-3'
21	S-1-R-781/802	5'-GTTGACAGTAGAGACAGTTGTA-3'
22	S-1-F-4472/4493	5'-AGGACAAGAAGGTAACCAAAGA-3'
23	S-1-F-4910/4928	5'-CCATCACGCGCTGTCGCGA-3'
24	S-1-R-6229/6248	5'-GTCGCCCAGTCAAAGGTGTC-3'

Fig. 1 List and positions of primers used in this study. The first and the last nucleotide positions (corresponding to the complete nucleotide sequence of porcine kobuvirus strain S-1-HUN) of the primers are in the primer names (X/X). Sequence-specific oligonucleotides were designed on the basis of the conserved regions of Aichi (AB040749) and bovine U-1 (AB084788) kobuviruses and the available overlapping S-1-HUN nucleotide sequences. *R* reverse and *F* forward in the primer names, *nt* nucleotide
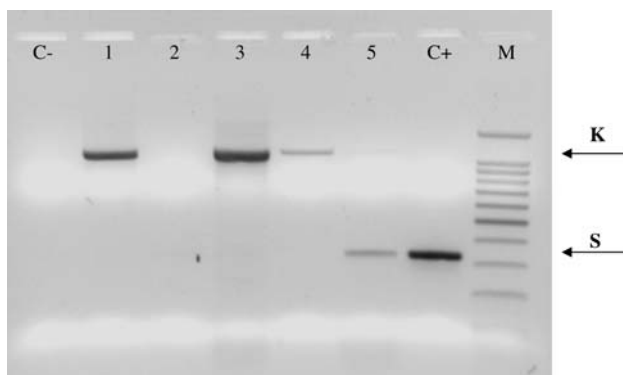
TGC, corresponding to nts 7491–7512 of U-1 virus) and UNIV-kobu-R (reverse, 5′-ATGTTGTTRATGATGGT GTTGA, corresponding to nts 7686–7707 of U-1 virus), amplifying a 216-nt region of the 3D protein. Amplicons were run in a 2% ethidium-bromide-stained agarose gel.

Sequence and phylogenetic analysis

PCR products were sequenced directly in both directions (at least twice) with BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Warrington, UK) using the PCR primers and an automated sequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems, Stafford, USA). Sequences were analysed using the ClustalX version 1.81 [24] and Genedoc version 2.6 programs [14]. A dendrogram was constructed using the neighbor-joining clustering method in MEGA (version 3.1) [13]. Possible recombination events in the coding region were analysed using Simplot software (version 3.5.1, http://sray.med.som.jhmi.edu/RaySoft/SimPlot). The secondary structures of the 5′- and 3′-terminal untranslated regions (UTR) were predicted using the Mfold program [29]. The complete sequence of porcine kobuvirus (strain Kobuvirus/swine/S-1-HUN/2007/Hungary) has been submitted to the GenBank/EMBL/DDBJ databases under accession number EU787450.

Results

Two (3.3%) of 60 samples were positive using p289/p290 primers for porcine sapoviruses. However, a ∼1100-nt-long PCR product was consistently found in 21 (35%) specimens (Fi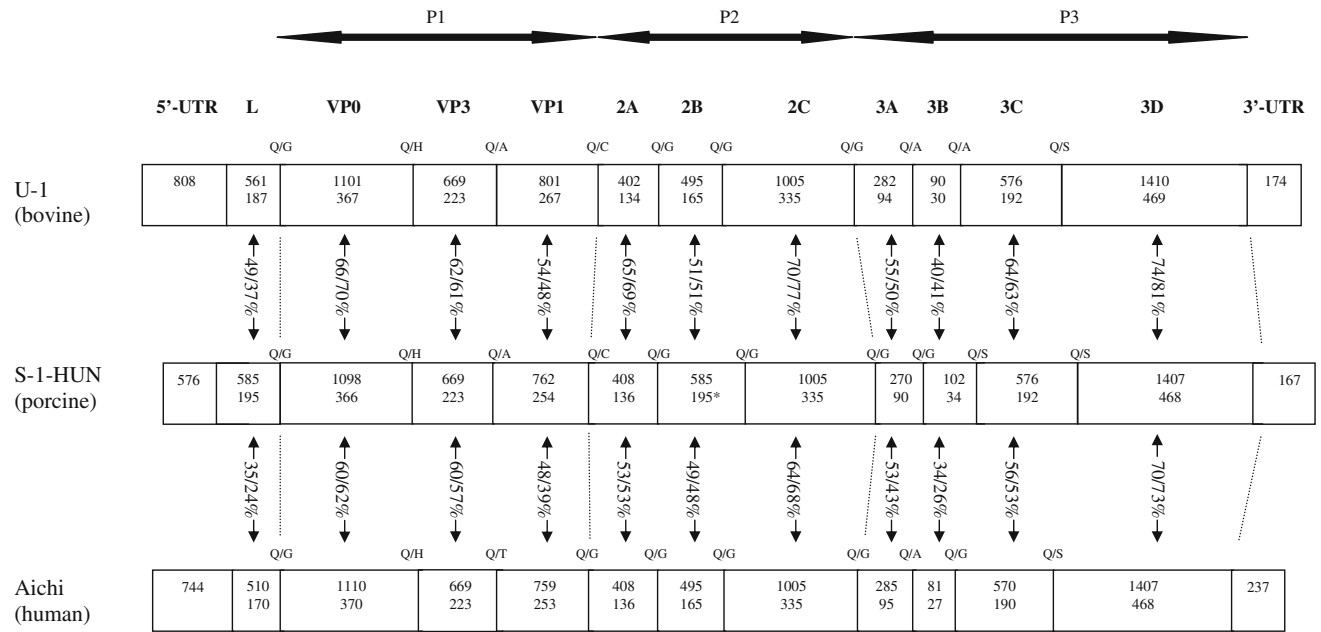g. 2). Nucleotide sequences of these non-specific PCR-products had genetic similarity to bovine (strain U-1) and human (Aichi virus) kubovirus $3C^{pro}$/3D RNA-dependent RNA polymerase genes in GenBank [20].

The complete RNA genome of the S-1-HUN strain consists of 8210 nt, excluding the poly(A) tail. A large ORF of 7467 nt, which encodes a potential polyprotein precursor of 2488 aa, was found, preceded at the 5′ end by 576 nt, and followed at the 3′ end by 167 nt and a poly(A) tail (Fig. 3). The base composition of coding region was found to be 20.6% A, 20.6% G, 31.5% C and 27.3% U. Possible cleavage sites for 3A/3B and 3B/$3C^{pro}$ were Q/G (Q/A in strain U-1) and Q/S (Q/A in strain U-1), respectively. Other cleavage sites were found to be identical to those in bovine strain U-1 (Fig. 3).

Analysis of S-1-HUN strain untranslated regions (UTRs)

The 576-nt-long 5′UTR was 168 and 232 nt shorter than those of Aichi virus (744 nt) and U-1 (808 nt), respectively. The secondary structure of the extreme 5′UTR (the first 108 nt) is very similar to those of kobuviruses, particularly (86%) to human kobuvirus (Aichi virus). It forms stem-loop (SL) domains with three potential stem-loops: SL-A, SL-B and SL-C (Fig. 4). Nine AUG motifs were identified in the 5′UTR at nts 215, 343, 355, 376, 387, 401, 515, 550 and 577. The last in-frame AUG is the possible authentic initiation codon, at position 577–579. This AUG codon is not preceded by a significant polypyrimidine tract. A longer pyrimidine tract (UUUCUCU) is located upstream in the RNA between nts 459 and 465, some 49 nts (spacer) upstream of the AUG motif at nt 515/516/517. The initiator AUG is located a further 59 nts downstream (at nt 577). This 59-nt region codes an AUG at position nt 550. In the GenBank database, sequence similarity (79% and 74%) was found between 3′ the end of the S-1-HUN 5′UTR region (from nt 496 to nt 549 and from nt 496 to 568) and the internal ribosomal entry sites (IRESs) of duck hepatitis virus 1 (EU395440) and porcine teschovirus (AY392537), respectively. Based upon these data and the predicted secondary structure of the 5′UTR IRES element, S-1-HUN has a potential *h*epacivirus/*p*estivirus-like (HP) type IV group-B-like internal ribosomal entry site (IRES IVB-like) with pseudoknot and conserved elements in an upstream bent hairpin-like structure (87-nt-long domains II with variant motifs in loop E: CAA versus AGUA) and in domain III (III_1, III_2, III_3, III_4, IIIa, IIIb, IIIc, IIId, IIIe and IIIf) (Fig. 4). The predicted lengths of the pseudoknot stem 1, stem 2, linker and spacer are 9, 8, 5 and 9 nucleotides, respectively. The conserved nucleotide motifs of branching hairpins IIId and IIIe are: GCGUGGCGGGAAUCC ACGC$_{489–508}$ and UACUGCUUGAUAGAGUC$_{518–534}$. The mispairing in helical segment III_1 (5′AGAG/5′UACU)

Fig. 2 Specific (S: 331 nt long) PCR product for porcine sapovirus (*lane* 5) and non-specific (K: ∼1100 nt long) PCR products for porcine kobuvirus (*lanes* 1, 3, 4, and 5) amplified by generic calicivirus primers p289/p290. Co-infection of porcine sapo- and kobuvirus is shown in *lane* 5. *C*− Negative control (porcine sapovirus), *C*+ positive control (porcine sapovirus), *M* 100-bp molecular marker

		P1				P2			P3			
5'-UTR	**L**	**VP0**	**VP3**	**VP1**	**2A**	**2B**	**2C**	**3A**	**3B**	**3C**	**3D**	**3'-UTR**
	Q/G	Q/H	Q/A	Q/C	Q/G	Q/G	Q/G	Q/A	Q/A	Q/S		
U-1 (bovine) 808	561 / 187	1101 / 367	669 / 223	801 / 267	402 / 134	495 / 165	1005 / 335	282 / 94	90 / 30	576 / 192	1410 / 469	174
	49/37%	66/70%	62/61%	54/48%	65/69%	51/51%	70/77%	55/50%	40/41%	64/63%	74/81%	
	Q/G	Q/H	Q/A	Q/C	Q/G	Q/G	Q/G	Q/G	Q/S	Q/S		
S-1-HUN (porcine) 576	585 / 195	1098 / 366	669 / 223	762 / 254	408 / 136	585 / 195*	1005 / 335	270 / 90	102 / 34	576 / 192	1407 / 468	167
	35/24%	60/62%	60/57%	48/39%	53/53%	49/48%	64/68%	53/43%	34/26%	56/53%	70/73%	
	Q/G	Q/H	Q/T	Q/G	Q/G	Q/G	Q/G	Q/A	Q/G	Q/S		
Aichi (human) 744	510 / 170	1110 / 370	669 / 223	759 / 253	408 / 136	495 / 165	1005 / 335	285 / 95	81 / 27	570 / 190	1407 / 468	237

* two copies of a 30-amino-acid (90 nucleotides) long tandem repeat in 2B region

Fig. 3 Genome organization of porcine kobuvirus S-1-HUN and comparison of the structure among human (Aichi) and bovine (U-1) kobuviruses. P1 represents viral structural proteins and P2 and P3 represent nonstructural proteins. Nucleotide (*upper number*) and amino acid (*lower number*) lengths are indicated in each gene box. Nucleotide and amino acid identity between S-1-HUN and members of the two other species of kobuviruses are indicated in percentage (nucleotide/amino acid %) in each gene. Predicted N-terminal cleavage sites are indicated at the top of the border in each gene box
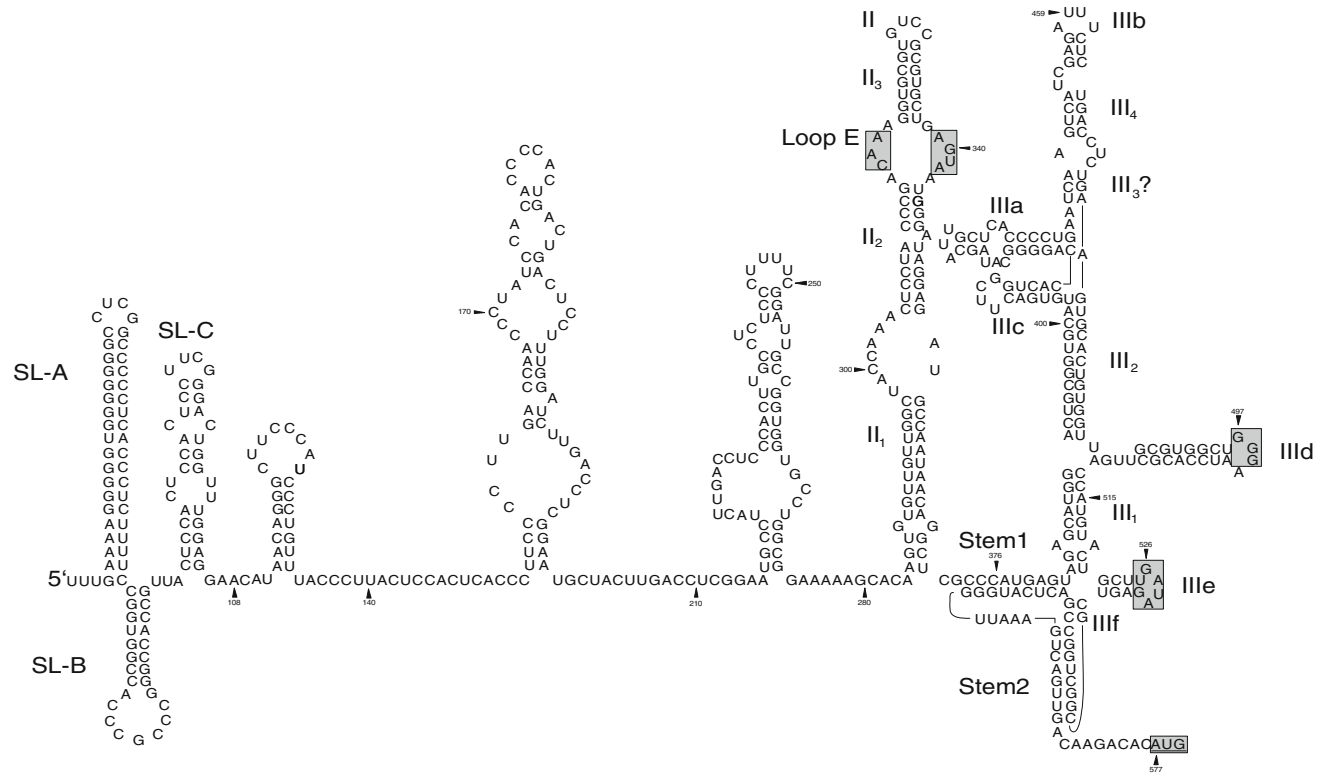
Fig. 4 Predicted RNA secondary structure of the porcine kobuvirus strain S-1-HUN 5′UTR as determined using the Mfold program. The three extreme 5′ terminal stem-loops (SL) are called SL-A, SL-B and SL-C [21], and the type-IV group-B-like IRES [6] has been annotated as previously proposed. Domains are labeled II and III; individual helical segments are labeled II₁, II₂, III₁, III₂, etc.; and individual hairpins are labeled IIIa and IIIb, etc. to maintain the continuity of the current nomenclature. The positions of conserved domains and the polyprotein AUG start codon are indicated by *shaded boxes*

is A–A in domain III (Fig. 4). The S-1-HUN strain 3′-UTR was 167 nt long and 70 nt and 7 nt bases shorter that of the Aichi virus and U-1 strain. No similar sequences were found in GenBank, but the predicted secondary structure of the 3′UTR of S-1-HUN consisted of three ds hairpin stems, analogous to U-1 strain (figure not shown).

Analysis of S-1-HUN strain coding regions

S-1-HUN has a leader (L) protein comprising 195 aa, which was 8 aa and 25 aa longer than those of the U-1 strain and Aichi virus, respectively (Fig. 3). The P1 polyprotein of strain S-1-HUN was 2529 nt long and 42 nt and 9 nt bases shorter that of U-1 strain and Aichi virus, respectively. VP0 and VP1 sequences varied in length: 366/367/370 aa for VP0 and 254/267/253 aa for VP1 in S-1-HUN, U-1 and Aichi virus, respectively (Fig. 3). The amino acid identity of the VP0, VP3 and VP1 proteins was 70%, 61% and 48% between S-1-HUN and U-1, respectively, and lower between S-1-HUN and Aichi virus (Fig. 3).

The P2 and P3 regions are 1998 nt/666 aa and 2355 nt/784 aa long, respectively. The amino acid identity of the P2 proteins (non-structural protein 2A–2C) is 68% and 59% to the U-1 strain and Aichi virus (Fig. 3). The amino acid sequence of the 2A region of the S-1-HUN strain had 69% and 53% identity with the U-1 strain and Aichi virus, and this conserved motif consisted of H-box/NC proteins (HWAL and NCXHFV) and a transmembrane domain. Within the 2B region, S-1-HUN encodes two copies of a 30-amino-acid (90-nucleotide)-long motif, AANRVAESI-ETTAS(/T)V(/A)VREADLARSTLNISM, one after the other (in tandem), which extends the 2B region (to 585 instead of 495 nucleotide in U1 or Aichi virus) (Fig. 3). The region 2C is conserved (68–77% aa identity) among the three kobuviruses. A highly conserved amino acid motif, GXXGXGKT (GPPGTGKS), in the nucleotide-binding domain of the putative picornavirus helicase was also found in the S-1-HUN 2C protein. The amino acid identity of the P3 proteins (non-structural proteins 3A–3D) is 71% and 63% to the U-1 strain and Aichi virus; however, the amino acid identity varied from 26% (Aichi virus 3A) to 81% (U-1 virus 3D) (Fig. 3). Protein 3A is smaller (90 aa versus 94 aa and 95 aa), and one copy of 3B (VPg) is longer in S-1-HUN than in U-1 and Aichi virus (34 aa versus 30 aa and 27 aa, respectively). A conserved picornavirus tyrosine residue was observed at the third aa position of S-1-HUN 3B (VPg). The 3C^{pro} protein (serine-type protease) contains a conserved picornavirus catalytic triad formed by histidine, aspartic acid and cysteine, found in S-1-HUN at aa 43, aa 86 and aa 145, respectively. A motif, GXCGG, that is conserved at the C terminus of enterovirus and rhinovirus 3C^{pro} was considered to form a
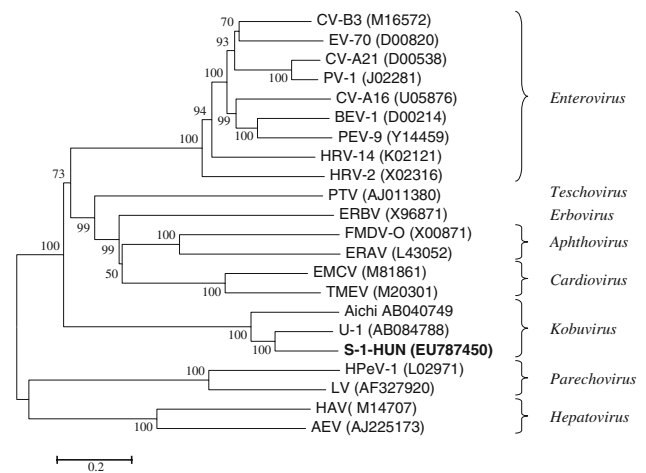
Fig. 5 Relationship between kobuviruses—including porcine kobuvirus (S-1-HUN, EU787450) *in bold*—and other picornaviruses based on amino acid sequences of picornavirus complete coding regions ("L", P1, P2 and P3). The phylogenetic tree was constructed by using neighbor-joining clustering method in MEGA version 3.1. Bootstrap values (based on 1000 replicates) for each node are given if >50%. The *scale bar* indicates amino acid substitutions per site. Reference strains were obtained from GenBank. CV-B3: coxsackievirus B3, EV-70: enterovirus type 70, CV-A21: coxsackievirus A21, *PV-1* poliovirus type 1, *CV-A16* coxsackievirus A16, *BEV-1* bovine enterovirus type 1, *PEV-9* porcine enterovirus type 9, *HRV-14* human rhinovirus type 14, *HRV-2* human rhinovirus type 2, *PTV* porcine teschovirus, *ERBV* equine rhinitis B virus, *FMDV-O* foot-and-mouth disease virus type O, *ERAV* equine rhinitis A virus, *EMCV* encephalomyocarditis virus, *TMEV* Theiler's murine encephalomyelitis virus, *Aichi* human Aichi virus, U-1: bovine kobuvirus, *HPeV-1* human parechovirus type 1, *LV* Ljungan virus, *HAV* hepatitis A virus, *AEV* avian encephalomyelitis-like virus

part of the active site, and this motif was present but altered to GMCGA in S-1-HUN (GLCGA in U-1 and GLCGS in Aichi virus). A histidine residue that probably participates in the substrate-binding pocket in the trypsine-like protease was found at aa 163 in S-1-HUN 3C^{pro}. The highly conserved amino acid motifs KDELR, YGDD and FLKR were also seen in 3D region of porcine kobuvirus.

No intergenotype recombination was observed in the kobuvirus coding regions. The highest sequence identity (up to 74%) was found in the 2C and 3D regions between S-1-HUN, U-1 and Aichi virus by Simplot. The phylogenetic relationships between the S-1-HUN and other representative picornavirus polyproteins (complete L, P1, P2 and P3 regions) are shown in Fig. 5. This confirmed that S-1-HUN is more closely related to kobuviruses, particularly to bovine kobuvirus, than to any other picornaviruses.

Detection of kobuviruses by a new generic primer pair

A 216-nt-long 3D region of Aichi virus (strain A846/88, kindly provided by T. Yamashita and strain Kobuvirus/human/Szigetvar-HUN298/2000/Hungary, FJ225407),

porcine kobuvirus (strain Kobuvirus/swine/S-1-HUN/2007/Hungary, EU787450) and bovine kobuvirus (strain Kobuvirus/bovine/Z20/2002/Hungary, FJ225406) was successfully amplified using kobuvirus primers UNIV-kobu-F/UNIV-kobu-R (3 min at 94°C, 40 cycles for 30 s at 94°C, 90 s at 42°C and 60 s at 72°C, and a final 10-min extension at 72°C) (figure not shown). Using these primers for re-amplification, 39 (65%) of the 60 fecal samples were positive for kobuvirus at the tested farm: 15 of 15 (100%), 14 of 15 (93.3%), 3 of 15 (20%) and 7 of 15 (46.7%) samples from swine under the age of 10 days, 3 weeks, 3 months and 6 months, respectively.

Discussion

Most picornavirus genera consist of two or more species [18]. This study reports on the genetic characterization and description of porcine kobuvirus, a new member of the genus *Kobuvirus* family *Picornaviridae*.

S-1-HUN has a typical picornavirus genome organization (VPg-5′NTR-leader protein-structural protein-nonstructural protein-3′NTR-poly(A) tail). Interestingly, genetic ambivalence was found in the S-1-HUN 5′UTR sequence. The nucleotide sequence of the first 108 nt and the secondary structure of the stem-loop domains (three stem-loops: SL-A, SL-B and SL-C) are very similar, particularly between porcine and human kobuviruses, and probably distinctive to kobuviruses. These data support the essential role of this region in the formation of virus particles and in RNA replication [21]. On the other hand, the genetic identity of the 3′ part of the 5′UTRs between strains S-1-HUN and U-1 and Aichi virus was very low. In addition, more genetic similarity and common motifs were found in the IRES element between porcine kobuvirus, porcine teschovirus [10] and duck hepatitis virus 1 than between porcine and human or bovine kobuvirus 5′UTRs. The secondary structures of the functionally important IRESs of human and bovine kobuviruses are not known [28]. By contrast, porcine kobuvirus S-1-HUN forms a potential *h*epacivirus/*p*estivirus-like (HP) type IV internal ribosomal entry site (IRES IV) described recently by Hellen and de Breyne as one of the new types of IRES among some picornaviruses [6]. Porcine kobuvirus S-1-HUN IRES is more closely related to type IV group-B-like IRESs; however, stem 1 is only 9 nt long (instead of 12 nt in a typical IVB-type IRES), which is characteristic for type-IV group-A IRESs [6]. Type-IV IRES elements described in members of the genera *Hepacivirus* (hepatitis C virus) and *Pestivirus* of the family *Flaviviridae* have also been identified in picornaviruses, including avian encephalomyelitits virus, duck hepatitis virus 1, duck picornavirus, porcine teschovirus, porcine entrovirus 8,

Seneca Valley virus and simian picornavirus [6]. These authors concluded that HP-like IRESs have been exchanged between unrelated virus families by recombination, which supports the hypothesis that RNA viruses consist of modular coding and noncoding elements that can exchange and evolve independently. Also, this type of IRESs initiates translation independently of eukaryotic initiation factors (eIFs) [6]. However, porcine kobuvirus S-1-HUN potentially belongs to the "HP IRES" group of viruses, which have conserved ribosome-binding regions in the pseudoknot and domains II and III (particularly hairpins IIId and IIIe). Further study is needed to provide biochemical evidence for this structure and the relationship between porcine kobuvirus and other kobuvirus IRESs. At this moment, it seems that members of the three kobuvirus species (human, bovine and porcine) contain genetically very different IRES sequences and probably form three different types of IRESs. Determination of whether the genetic similarity of porcine kobuvirus IRES can be attributed to recombination events and why this region is a potential promiscuous region (independent genetic entity) must await a detailed study including more kobuvirus field isolates and 5′UTR sequences. Co-infection was tested, and the sample used for this study was negative for porcine teschovirus by RT-PCR [16].

The major differences between kobuviruses and other picornaviruses are found in the coding region of the L protein, the absence of a VP0 cleavage site and a distinct form of the 2A protein [22]. The S-1-HUN strain probably also exhibited these features; however, PAGE analysis of purified capsid proteins including the possible intact VP0 was not done. Amino acid identities between S-1-HUN and U-1 and Aichi viruses are lower in the L protein than 3 in A and higher than in 3B. However, the porcine kobuvirus L protein is more closely related to bovine kobuvirus than to the human kobuvirus L protein; relatively low similarity has been found (with two potential conserved motifs: PEDxLxDS and LPG) between the three viral L proteins. The function of the kobuvirus L protein is not known. Neither the catalytic dyad (Cys and His), conserved in a papain-like thiol protease and found in foot-and-mouth disease virus L protein [5], nor a putative zinc-binding motif, Cys–His–Cys–Cys, found in encephalomyocarditis virus [3], could be identified.

The percentage identity of the VP1 protein between S-1-HUN and Aichi or U-1 strains was lower than that of the VP0 region. VP1 is the most exposed and immunodominant part of the picornavirus capsid proteins [28], and this protein is also the most variable structural protein among the three kobuviruses. In a previous study, antibody to Aichi virus was not detected in pigs and cattle, and conversely, antibody to bovine U-1 strain was not detected in human and pig sera [28]. This raises the possibility that

porcine kobuvirus forms not only a distinct genetic but also a different antigenic type (serotype). Further study is necessary to determine the antigenic relationship between the three kobuviruses.

A high degree of amino acid difference has been identified among 2A proteins of picornaviruses. Some picornaviruses (parechoviruses, avian encephalomyelitis virus) including kobuviruses contain conserved motifs (H-box/NC) that are characteristic of a family of cellular proteins (H-rev107) involved in control of cell proliferation [7]. Like U-1 and Aichi virus, S-1-HUN also encodes this H-box/NC motif in the 2A protein.

A tandem repeat (two consecutive copies of a 30-aa-long motif) was detected within the 195-aa-long non-structural protein 2B (also called viroporin in enteroviruses). The nucleotide and amino acid similarities were 82% and 93%, respectively, between the two repeated sequences. Among picornaviruses, tandem repeat regions are known in aphthovirus (three copies of complete 3B); however, two unrelated 2As are found in seal picornavirus and duck hepatitis virus [11]. Interestingly, besides the genetic similarity of one copy of this 2B sequence motif of S-1-HUN to those of bovine (61%) and human (56%) kobuviruses, the repeated sequence has a genetic similarity (23%) to the C-terminus of the cellular IQ-motif-containing GTP-ase activating protein 2 (IQGAP2) detected only in liver [2], according to a BLAST search in GenBank. Homologues between human and picornavirus sequences are not unique. The entero/rhinovirus 3C^{pro} protein (a cellular trypsin-like serine protease), the aphthovirus L protein (papain-like protease), and the parecho/kobuvirus 2A protein (H-box/NC, see above) have cellular homologues [7]. Further analysis is needed to find out whether this observation with kobuvirus 2B and IQGAP2 represents a significant similarity. The kobuvirus 2B function is not known; however, protein 2B has a different function in members of different picornavirus genera [4].

Low sequence similarity was detected among the three kobuviruses in 3′UTR region. In addition, no 3′UTR sequences similar to that of S-1-HUN were found in the GenBank database. Still, the predicted secondary structure of this relatively long non-translated region consisted of three ds hairpin stems with analogy to those in the U-1 strain.

An atypical genome base composition has been identified in Aichi and U-1 viruses [28]. The pyrimidine content (31.5% C and 27.3% U) of S-1-HUN is similar to that of the U-1 strain and is also higher than that of other picornaviruses. The high C composition in the genome is suspected to be a typical trait of kobuviruses [28].

More than 60% of the apparently healthy pigs at the farm carried the virus. The incidence was 90% under the age of 3 weeks. These results indicate a general circulation and probably persistent infection of kobuvirus on the tested farm. On the other hand, based upon the analogy of the pathogenesis of many picornaviruses and the first detection of bovine kobuvirus in culture medium derived from cattle sera [28], we cannot exclude the possibility that kobuviruses in general, and S-1-HUN-like kobuvirus in swine, can cause viraemia (and generalized infection) and have target organ(s) inside the body. This hypothesis should be taken into consideration, too, in kobuvirus pathogenesis—which of course needs confirmation—since Yamashita and his co-workers suspect that culture medium supplemented with cattle sera had possibly been contaminated with cattle feces containing bovine kobuviruses [28]. S-1-HUN-like virus may typically be asymptomatic in swine; however, more epidemiological studies are required regarding the significance of this virus as a causative agent in any diseases in domestic pig and other animals.

The screening primer pair (6261/6779) designed for human kobuvirus 3C/3D junctions [27] was negative for porcine kobuvirus. The kobuvirus primers (UNIV-kobu-F/R) designed in this study gave PCR products for all three (human, bovine and porcine) known kobuviruses. The sensitive kobuvirus primers designed based upon the conserved 3D sequences of human, bovine and porcine kobuviruses give an opportunity for successful molecular epidemiological studies for the detection of kobuviruses in these species (in one test reaction) and probably in further animal species.

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