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Genome comparison of a novel foot-and-mouth disease virus with other FMDV strains

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Abstract

The genome of a novel foot-and-mouth disease virus, HKN/2002, was 8104 nucleotides (nt) in length (excluding the poly(C) tract and poly(A) tail) and was composed of a 1042-nt 5′-untranslated region (UTR), a 6966-nt open reading frame, and a 93-nt 3′-UTR. Genome sequences of HKN/2002 and other known FMDV strains were compared. The VP1, VP2, and VP3-based neighbor-joining (NJ) trees were divided into distinct clusters according to different serotypes, while other region-based NJ trees exhibited some degree of intercross among serotypes. Mutations in HKN/2002 were revealed, including frequent deletions and insertions in the G–H loop of VP1, and deletion involving 10 amino acid residues in the 3A protein. An evolutionary relationship of HKN/2002 with an Asian FMDV lineage isolated from a Hong Kong swine host in 1970 was postulated. A 43-nt deletion identified in the 5′-UTR of HKN/2002 possibly contributed to the loss of one pseudo-knot domain.

Keywords: Picornavirus; Serotype; Phylogenesis; Sequence; Alignment

Foot-and-mouth disease (FMD) is a highly contagious disease inflicting cloven-hoofed animals. The disease leads to significant economic losses due to death of young animals, decreased productivity, and trade sanctions against livestock and animal products from infected regions. FMD virus (FMDV) is a positive sense, single-stranded RNA virus of the family *Picornaviridae*, genus Aphthovirus. In addition to categorizing into seven serotypes (A, O, C, Asia I, SAT 1, SAT 2, and SAT 3) [11], FMDVs can be genetically classified based on their geographic origin (topotypes); e.g., the serotype O can be grouped into eight topotypes [Cathay, Middle East-South Asia (ME-SA), South-East Asia (SEA), Europe-South America (Euro-SA), Indonesia-1 (ISA-1), Indonesia-2 (ISA-2), East Africa (EA), and West Africa (WA)] based on nucleotide differences of up to 15% [42].

The FMDV genome contains over 8000 nucleotides, with a 5′ terminus covalently bound to a small viral polypeptide VPg (3B), and a 3′ poly(A) tail [12]. The genome contains a long open reading frame (ORF) translated into a single polyprotein, that can be cleaved into four structural proteins (VP1, VP2, VP3, and VP4),

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and 10 non-structural proteins (L, 2A, 2B, 2C, 3A, 3B1, 3B2, 3B3, 3C, and 3D) [19]. The icosahedral symmetrical viral capsid is assembled from the four structural proteins [1], with VP1 being the most important because of its dual function in cell receptor binding and antigenic determination [34,38,45]. Currently, much research effort has been focusing on the sequence analysis of structural proteins, especially VP1, owing to their dominant roles in antigenic/serotype determination [3,24,30,41]. The non-structural proteins are not directly involved in antigenic/serotype determination. L, 2A, and 3C are the major proteinases for hydrolytic cleavage of the polyprotein [25,40,43]. 3B (VPg), also known as the genome-linked protein, may be involved in priming RNA replication [15,47]. 3D, the viral RNA-dependent RNA polymerase (RdRp), is responsible for both positive- and negative-sense RNA replication. The recently resolved crystal structure of the poliovirus 3D and other genetic/biochemical analyses suggest that the polymerase might function as a higher order oligomeric structure [20,22]. 3A has been found to be associated with host altering [4,17,26]. The functions of other non-structural proteins remain unclear.

A novel FMDV strain, HKN/2002, was isolated from swine hosts during an FMD outbreak in Hong Kong in February 2002. The sequences of the VP1 [14] and 3D [8] genes of this strain were reported previously. This report will further analyze the complete genome sequence of HKN/2002. The gene and deduced amino acid sequences of the structural and non-structural proteins of HKN/2002 are compared with other FMDV sequences. The sequence analyses may provide insight into the phylogenetic origin of new FMDV strains, important to host recognition and virulence.

Materials and methods

Cloning of the HKN/2002 full-length sequence. The HKN/2002 strain was generously provided by the Agriculture, Fisheries, and Conservation Department, Hong Kong SAR, China. Total RNA of FMDV-infected BHK-21 cells was extracted using RNAgents Total RNA Isolation System (Promega, Madison, WI, USA). Reverse transcription was carried out using five antisense genome specific primers (Rc, R5, R1, R66, and R2; Table 1) and Superscript II RT RNase H^- (Invitrogen, Carlsbad, CA, USA). Five cDNA fragments corresponding to nt 1–371, 372–1983, 1920–4245, 4214–6658, and 6599–8104 (according to the HKN/2002 genome sequence) were amplified by PCR using five primer sets, F1–Rc, Fc–R5, F5–R1, F2–R66, and F4–R2, respectively. The primers shown in Table 1 were based on previously published FMDV sequences, therefore some primer sequences may not be identical to that of HKN/2002. The resultant PCR products were cloned using the pGEM-T easy vector system (Promega), and the DNA sequence for each clone was determined.

Sequence analysis. Sequences were obtained from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) and the OIE/FAO World Reference Laboratory for Foot-and-Mouth Disease (http://www.iah.bbsrc.ac.uk/virus/Picornaviridae/Aphthovirus/index.html). The sequences used for each gene were firstly examined to exclude ambiguous sequences, which were incomplete, frame-shifted, or contained N in the sequences. Multiple alignments were performed by a ClustalX multiple sequence alignment program 1.83 [44]. The neighboring-joining (NJ) tree was constructed based on the result of alignment by MEGA 2.1 [28].

Results

Full-length genomic sequence of HKN/2002

The 8104-nt HKN/2002 genome contains a 5′-UTR of 1042 nt (excluding the poly(C) tract), an open reading frame (ORF) of 6966 nt, encoding a polyprotein of 2322 or 2294 amino acid (a.a.) residues due to two alternative initiation sites separated by 84 nucleotides, and a 3′-UTR region of 93 nucleotides (excluding the poly(A) tail). The genome sequence has been submitted to the NCBI (GenBank Accession No. AY317098).

HKN/2002 belongs to serotype O

Sequence analysis of HKN/2002 with 26 other reported FMDV genomes indicates that the novel HKN/2002 strain is of serotype O. Fig. 1 shows a neighboring-joining (NJ) tree constructed based on the sequence alignment of these 27 genomes, which are distinctly divided into five serotypes. HKN/2002 is tightly clustered

Table 1
Primers used in cloning the full-length HKN/2002 genome

Primer	Position	Sequence	Orientation
F1	1	GGG GGA TCC TTG AAA GGG GGC GCT AGG	Sense
Rc	371	NNT GGG GGG GGG G	Antisense
Fc	372	NNA CCC CCC CCC CCC	Sense
R5	1983	GAC TGG GTT GTC GAG GTC GTG	Antisense
OF6	1920	CTA CCC TCC TCG AGG ACC GC	Sense
R1	4245	AAG AGA CTG GAG AGC GAG TCG GAG ATC TTC	Antisense
F2	4214	AAG AAG ATC TCC GAC TCG CTC TCC AGT CTC	Sense
R66	6658	CTT GGT TTT GCG CAT CAC GTG GAC G	Antisense
F4	6599	GGG TTG ATC GTT GAT ACC AGA GA	Sense
R2	8104	GGG GCG GCC GCG GAT TAA GGA AGC CGG GAA AGC CC	Antisense

Artificially added nucleotides are underlined; N indicates A or C or T or G.

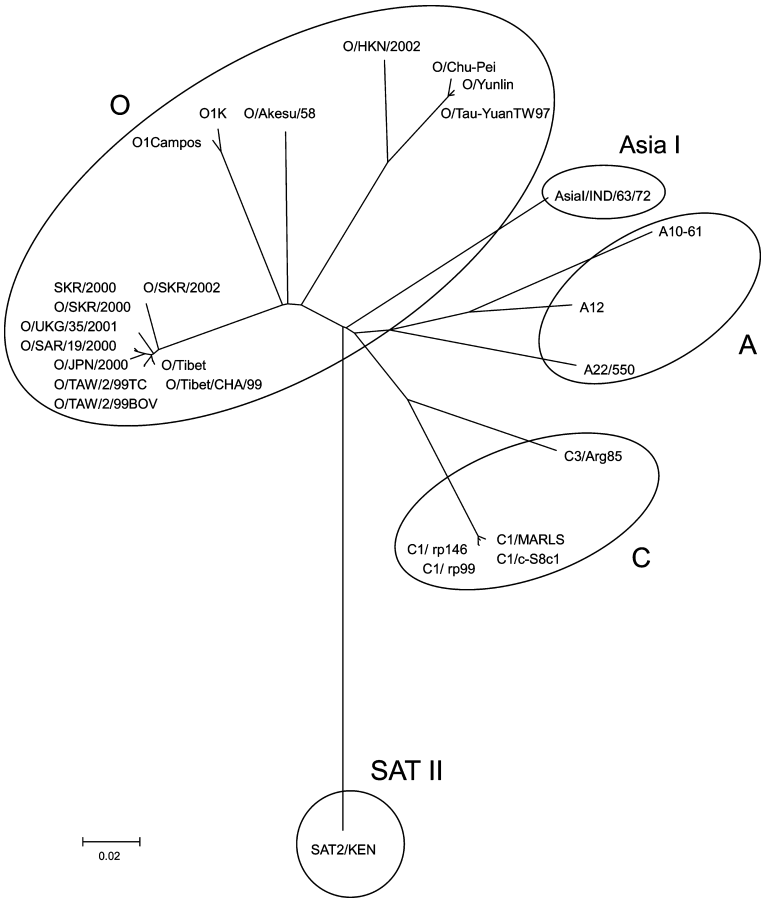

Fig. 1. A neighboring-joining tree based on 27 FMDV genome sequences. The GenBank accession numbers of the 27 genomes are listed as following: O1Campos, AJ320488; O/Akesu/58, AF511039; O/Tibet, AF506822; O/Tibet/CHA/99, AJ539138; O1K, M35873, X00871; O/HKN/2002, AY317098; O/JPN/2000, AB079062, AB079061; O/SAR/19/2000, AJ539140; SKR/2000, AJ539139; O/SKR/2000, AY312586, AY312587; O/SKR/2002, AY312588, AY312589; O/Tau-YuanTW97, AF154271; O/Chu-Pei, AF026168; O/Yunlin, AF308157; O/TAW/2/99TC, AJ539136; O/TAW/2/99BOV, AJ539137; O/UKG/35/2001, AJ539141; A10-61, M14409, X00429; A12, L11360, M10975; A22/550, X74811, X74812; C3/Arg85, AJ007347; C1/c-S8c1, AJ133357; C1/rp99, AJ133358; C1/rp146, AJ133359; C1/MARLS, AF274010; AsiaI/IND/63/72, AY304994; and SAT2/KEN, AJ251473.

in the O serotype and closely linked to three Taiwan isolates, O/Tau-YuanTW97, O/Yunlin, and O/Chu-pei (from a major outbreak in Taiwan in 1997), with genetic distances of 0.066, 0.067, and 0.069, respectively (distance matrix data not shown). The close link between HKN/2002 and these three Taiwan isolates is consistent with our previous finding using the VP1 sequence [14].

Sequence conservation varies in different regions of the FMDV genome

The nucleotide and deduced a.a. sequences of each region (5′-UTR, L, VP4, VP2, VP3, VP1, 2A, 2B, 2C, 3A, 3B, 3C, 3D, and 3′-UTR) of HKN/2002 and other FMDV strains were compared (Table 2). NJ trees were constructed based on sequence analyses (Fig. 2 shows the VP1, VP2, VP3, and 3A-based NJ trees. Other region-based NJ trees are not shown). In general, the non-structural protein regions are more conserved than the structural protein regions, with 3D exhibiting the highest conservation (94–99% similarity in a.a. sequences), and VP1 showing the lowest conservation (45–95% similarity). In the non-structural protein regions, the L (61–95%) and 3A (68–96%) genes are exceptionally divergent. The structural protein, VP4, is exceptionally conserved (91–100% similarity). The 5′-UTR (78–95%) is more conserved than the 3′-UTR (78–95% versus 75–90%; Table 2).

Only VP1, VP2, and VP3-based NJ trees are consistent with serotypes

Among the region-based NJ trees, only the VP1 [14], VP2, and VP3-based trees (Figs. 2 A, B, and C) are distinctly divided into clusters of different serotypes, corresponding to their roles in antigenic determination. The eight topotypes of serotype O are marked on the VP1-based NJ tree (Fig. 2A), and the novel HKN/2002 strain belongs to the Cathay topotype. For the NJ trees of other regions, although the branches are mainly divided

Table 2
Sequence comparison of HKN/2002 with other strains in each gene region

Regions	nt sequence similarity (%)		Amino acid sequence similarity (%)	
	Minimum	Maximum	Minimum	Maximum
5′-UTR	78	95	—	—
L	63	93	61	95
VP4	80	95	91	100
VP2	67	92	70	97
VP3	63	92	59	99
VP1	51	94	45	96
2A	81	98	94	100
2B	87	96	90	100
2C	86	94	90	98
3A	69	97	68	96
3B	87	99	92	99
3C	78	93	85	100
3D	86	94	94	99
3′-UTR	75	90	—	—

All the complete sequences of each gene region from NCBI are used in comparison. Sequence data for each gene are available upon request.

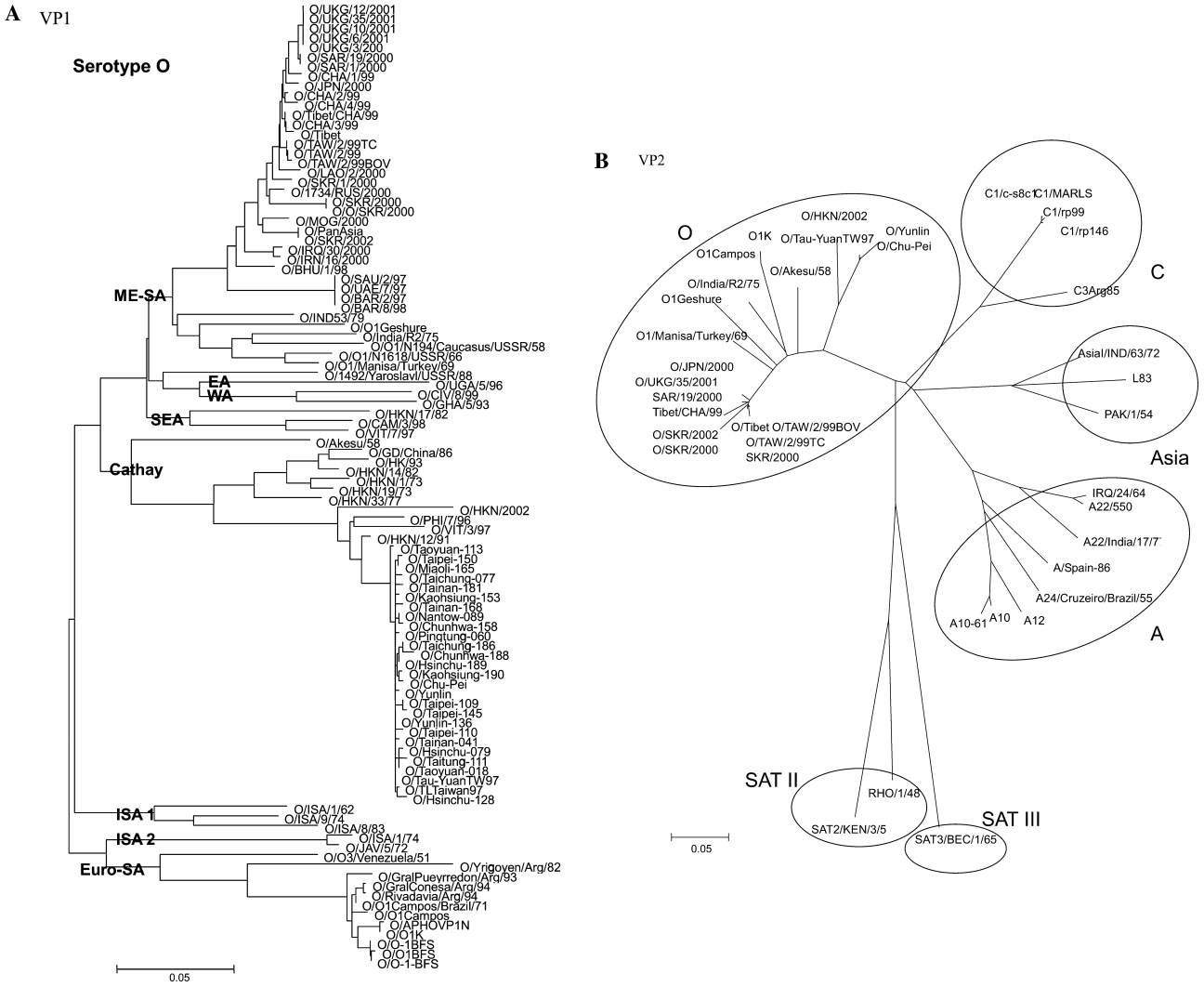

Fig. 2. VP1, VP2, and VP3-based NJ trees. The VP1-based NJ tree shows the O serotype and topotypes.

according to serotypes, a few intercrosses are present. These intercrosses occur either in the same or adjacent geographical regions or among countries that have international trade relationship (data not shown).

Sequence alignment of each region reveals mutations in VP1, 3A, and 5′-UTR

Frequent deletions/insertions are found in the G–H loop of VP1 according to a comparison of the a.a. sequences of 352 VP1s, and these deletions/insertions can be grouped into different serotypes suggesting their role in antigenic determinant. Interestingly, four natural RGD mutants, O/Akesu/58 (GenBank Accession No. AF511039), O3/Venezuela/51 [31], A27 [46], and A10-61 [7] are revealed during comparison, possessing residue, S, I, A, and S instead of R, respectively. The existence of these natural RGD mutants suggests the variability of FMDVs in host cell binding [2,48].

A 10-a.a. deletion (position 93–102) in the 3A protein of HKN/2002 is evident (Fig. 3). A similar deletion was previously discovered in a swine-hosted Asia lineage, the O/HKN/21/70 strain [4,26]. HKN/2002 was isolated from a severe FMD outbreak of swine in Hong Kong. Considering its porcinophilic property and geographic location, it is highly likely that HKN/2002 is a novel descendent of this Asia lineage. The 3A-based NJ tree (Fig. 2D) indicates that HKN/2002 is indeed closely related to this Asia lineage. Mutations accumulated in O/HKN/21/70 from 1970 to 2002, as shown in Fig. 3, also support this speculation. Among all sequences derived from the O/HKN/21/70 strain, HKN/2002 shares the highest homology with O/HKN/7/96 (96% similarity in amino acids), and the lowest homology with O/HKN/21/70 (87% similarity in amino acid) (Data not shown). We also notice that the three Taiwan strains all belong to this Asia lineage, suggesting their close relationship with HKN/2002. Moreover, an 11-a.a. deletion (position 134–144) in 3A can be observed in O/CAM/1/98, O/CAM/2/98, O/CAM/3/98, O/CAM/11/94, O/CAM/12/94, and O/VIT/2/97. Unlike the O/HKN/21/70 strain, this particular deletion does not appear to associate with any particular host species [26].

Comparison of the 5′-UTR (Fig. 4) shows a 43-nt deletion close to the 3′-end of the poly(C) tract in HKN/2002 and serotypes A, O, C, and Asia 1. The 43-nt deletion is located in a region containing pseudoknot (PK) structures in serotypes A, O, and C [9,13,29,39], and this deletion may cause the loss of one PK domain. Most FMDV strains have four PK structures, PK I, II, III, and IV sequentially, located at the 3′-side of the poly(C) tract. The structure of each

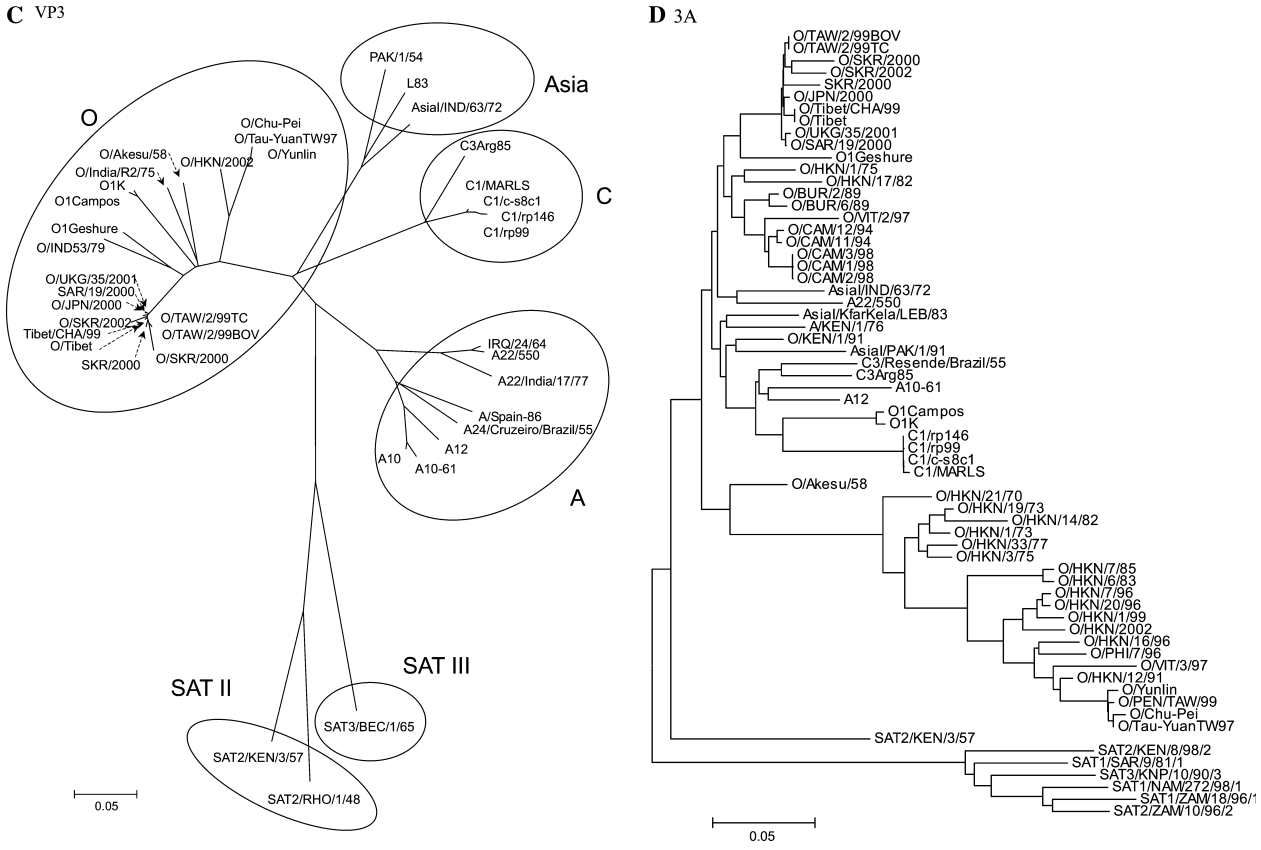

Fig. 2 (*continued*)

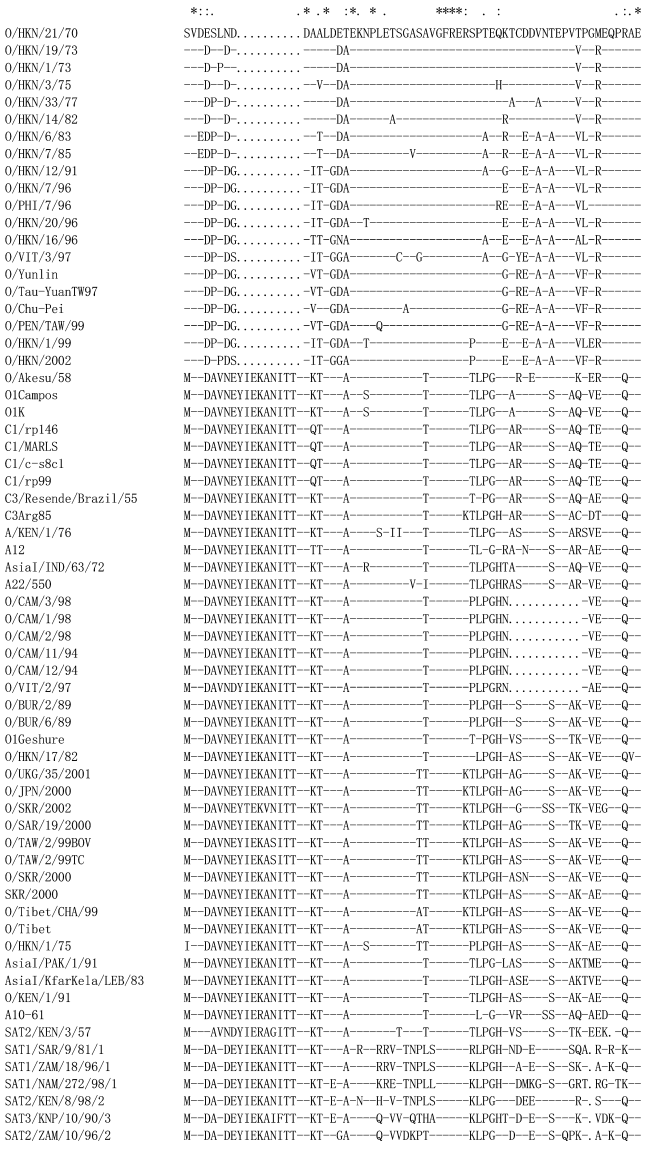

Fig. 3. Alignment of amino acid sequences of 3A protein (from position 85–153). The alignment was produced using ClustalX 1.83. (*) Residues conserved across all compared sequences. (:)(.) See ClustalX 1.83 for details.

PK is similar; a 4-nt stem 1 (S1), a 3–5-nt stem 2 (S2), and a 5–7-nt loop, together with an 18–20-nt highly conserved spacing between two adjacent PKs [9,39]. The function of PKs is not clear, but it is assumed that the PK structures, together with the S fragment and the poly(C) tract, play a role in genome replication [33]. The deletions in the PK regions are common in the evolution of FMDV, and the loss of one or two PKs has been reported [13].

Our data indicate that the 43-nt deletion exists in 11 strains including serotypes A, O, C, and Asia 1, isolated from swine and bovine sources. The predicted PK structure in HKN/2002 is illustrated in Fig. 5. Sequence analysis of the PK domains in various FMDV strains indicates that the conservation differs in four PKs (Table 3). The sequence and position of PK IV are identical in S1 for all strains analyzed, while each of the other three PKs can be divided into several groups according to their nucleotide sequences of S1. It is interesting that mutations in some serotypes O may demolish the S1 region of PK I and II. The VP1-based NJ tree shows that these strains belong to a PanAsia strain of the Middle East-South Asia (ME-SA) topotype [27].

Discussion

Higher gene sequence conservation of non-structural proteins

In general, non-structural proteins, crucial roles for viral propagation, are more conserved than the structural proteins. Most mutations or deletions in the non-structural proteins could be detrimental to viral replication and protein processing. Converged mutations in structural proteins may help FMDV to evade immune response mounted by the host, while maintaining the functional capsid. Consequently, the gene sequences of the structural proteins could mutate with a higher frequency for retaining evolutional advantages. But exception to this generality exists: the high divergence of the leader protein, L, exists in the N-terminus of laboratory (synthesized from the first AUG) [16], a region involved in start codon selection during translation [32]. Since this region is not essential for proteinase activity [35], its unusual variation may reflect its role in either RNA–RNA or RNA–protein interaction that specifically enhances IRES-dependent translation [16]. The low conservation of the non-structural protein 3A could be due to its putative role in the adaptability of the virus to the host, while the high conservation of the structural protein, VP4, is consistent with the fact that VP4 is embedded inside the capsid and thus contributes little to the viral antigenicity [1,5,10].

Strategy for host cell binding—RGD mutants

The existence of four natural RGD mutants, O/Akesu/58, O3/Venezuela/51, A27, and A10–61, provides new insight into the general understanding of the molecular basis of FMDV in host cell binding. The highly conserved arginine–glycine–aspartate (RGD) sequence located in VP1 mediates binding of FMDV to the cellular receptor, the RGD-binding integrins [6]. Since these four strains lack the RGD motif, host cell recognition may be mediated through another integrin receptor or a non-integrin pathway. Cell culture-adapted FMDV could use heparan sulfate (HS), a cell surface glycosaminoglycan, as a cellular receptor [23]. Artificially passaged FMDV lacking the RGD motif is able to use a third

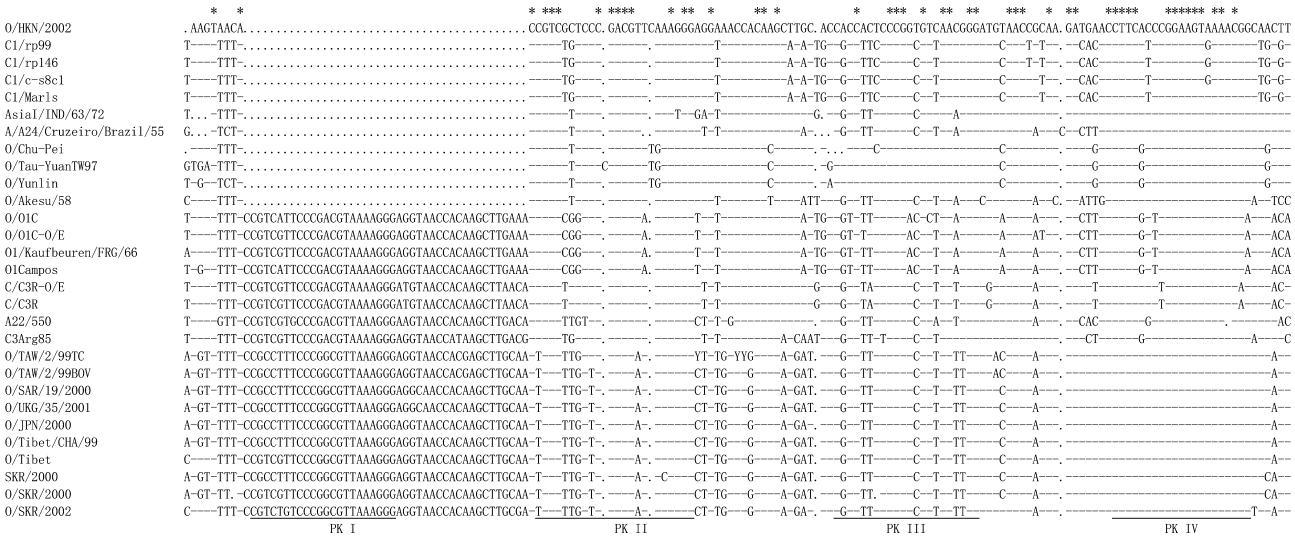

Fig. 4. Alignment of nucleotide sequences of 5′-UTR. Displayed sequences are the predicted PK structure region, starting from the 3′ side of poly(C) tract. Positions of each PK are shown.

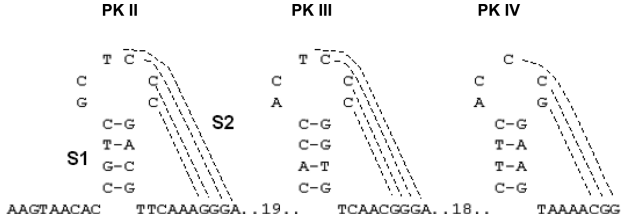

Fig. 5. Predicted pseudo-knot structures in the 3′ side of poly(C) tract region of HKN/2002. The broken lines indicate the interactions in S2. The nucleotides between each pseudo-knot are shown as the number of nucleotides only. The complete sequences are given in Fig. 4. PK I is defective in HKN/2002.

receptor (neither integrin-based nor HS) for entry into the host cell [2]. In fact, an FMDV strain carrying KGE, instead of RGD, is able to induce mild disease in pigs, and maintain its KGE motif [48]. Evaluation of these KGE mutants indicates that their growth in culture is not dependent on HS. These mutants may have a similar type of integrin that is utilized by the wild-type viruses (with RGD motif), via the four residues of VP1 surrounding the pore on the five axes of the capsid. Currently, only four, out of 356 strains examined (1.1%), are revealed to lack the RGD motif. The dominance of the RGD sequence suggests its advantages in host cell recognition and binding.

Changes in 3A are associated with host range determinants

Most FMDVs have 3A proteins of 153 a.a., larger than those of other picornaviruses (e.g., 87 a.a. for poliovirus 3A). Changes in the 3A protein have been associated with altered host range in other genera of picornaviruses, such as hepatoviruses [18], rhinoviruses, and enteroviruses [21]. A 19- or 20-a.a. deletion in the C-terminus of the 3A protein was found in the attenuated, egg-adapted FMDV of bovine origin [17]. A similar 10-a.a. (position 93–102) in a swine-hosted Asia lineage has been described in previous section. The deletions in the 3A protein are mainly found in viruses isolated from altered or specified host, however, it is difficult to evaluate the importance of these 3A deletions in host alteration since the same deletions could also be observed in a few viruses isolated from normal hosts [26].

An 11-a.a. deletion (position 134–144) in 3A found in six viruses isolated from cattle and pigs (Vietnam and Cambodia) (Fig. 4) has not demonstrated association with particular host species, although the number of viruses identified in this group is quite low. But this deletion appears to arise more recently (O/CAM/11/94, isolated in 1994) than the 93–102 10-a.a. deletion found in the O/HKN/21/70 group of viruses. Interestingly, the O/CAM/11/94 group is closely clustered with a virus isolated from pigs in 1989 (Burma), O/BUR/6/89, which contains intact 3A regions [26].

Intact 3A protein without deletion but undergoing a single a.a. substitution may mediate the adaptation of a serotype C to a guinea pig host [36], suggesting a

Table 3
Sequences and numbers of nucleotides in predicted pseudo-knot structures

PK	Base pairs in S1	Nt No. in loop	Strains
I	CGTC···GACG	6	Most strains
	CGCC···GGCG	6	O/TAW/2/99BOV
			O/TAW/2/99TC
			O/JPN/2000
			O/SKR/19/2000
			O/UKG/35/2001
			O/SKR/2000
			O/Tibet/CHA/99
	CG**T**C···G**G**CG	6	O/CHA/1/99
			O/SKR/2002
II	CGTC···GACG	6	Most strains
		7	O/Tau-YuanTW97
	TGTC···GAC**G**	6	O/TAW/2/99BOV
			O/TAW/2/99TC
			O/JPN/2000
			O/SKR/19/2000
			O/UKG/35/2001
			O/SKR/2000
			O/Tibet/CHA/99
			O/CHA/1/99
			O/SKR/2002
III	CGCC···GGCG	6	Most strains
	CGTC···GACG	6	O/O1C-O/E
			O/O1C
			O1K
			O1Campos
	CACC···GGTG	6	O/HKN/2002
			O/Chu-Pei
			O/Yunlin
			O/Tau-YuanTW97
IV	CTTC···GAAG	5	All strains

Sequences in S1 are shown from the 5′ to 3′ terminal. Bold letters indicate unpaired nucleotides that will destroy the original stem structure.

potential role of point mutations in host range determinants. Likewise, by comparing the genomic sequences of two viruses in Korea, O/SKR/2000, and O/SKR/2002, isolated from cattle and pigs, respectively, substitutions (but not deletion) of eight a.a. could be found in the 3A region [37].

PK structure may be involved in virulence

The mutations in PK I and II found in a PanAsia strain imply a possible relationship between PK structure and the viral virulence. The PanAsia strain was first isolated in northern India in 1990 and has rapidly spread to the Middle East, Europe, South-East Asia, and South Africa by 2001. This strain had caused devastation in livestock trade and countless economic losses [27]. This strain seems to persist after out-competing all other previously established strains, therefore pursuing the functional significance the PK mutations is worthwhile. Mutations in the PK I and II domains of the PanAsia strain might contribute to its unusual severe virulence by enhancing the genome replication through an unknown mechanism.

In summary, sequence analysis of a novel FMDV, HKN/2002, of the Cathay topotype (serotype O) has been described. A 10-a.a. deletion in the 3A protein, and a 43-nt deletion in the 5′-UTR of HKN/2002 may be involved in the processes of host alteration and virulence.

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