Evolution and molecular epidemiology of foot-and-mouth disease virus in China

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Foot-and-mouth disease (FMD), caused by foot-and-mouth disease virus (FMDV), is considered one of the most important viral diseases of cloven-hoofed animals, causing severe economic losses in affected regions of the world. Three serotypes (A, O, and Asia 1) of FMDV have been identified in China since 1958. In addition, the occurrence of novel subtypes within these serotypes has made the epidemiology of FMDV more complicated over the last few years. In this review, we summarize the history and the current epidemiological situation in China, genetic diversity (e.g., quasispecies dynamics, antigenic heterogeneity, and functional constraints), intertypic recombination, and the evidence for positive selection of different FMDV serotypes. We also assess these genetic data to understand the origin, evolution, and transmission of FMDV, the findings of which may be useful in developing control measures for future epidemics.

Foot-and-mouth disease virus, phylogenetic analysis, recombination, positive selection

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Foot-and-mouth disease (FMD) is an acute, highly contagious disease of cattle, pigs, sheep, goats, and many cloven-hoofed wild animals [1]. Despite low mortality rates, FMD causes significant economic losses because of the negative effects livestock production and international trade restrictions on animals and animal products [2]. Foot-and-mouth disease virus (FMDV), the etiological agent, is a positive-strand RNA virus that belongs to the genus Aphthovirus of the family Picornaviridae. There are 7 distinct serotypes of FMDV (A, O, C, Asia 1, and South African territories [SAT] 1–3) and multiple subtypes reflecting significant genetic variability in each serotype [3–5]. Despite intensive research efforts, FMDV still remains enzootic in many regions of the world. It is endemic and extensive in some parts of Asia, Africa, and sporadic in certain member states of the European Union [6–10].

In China, 3 of the 7 serotypes of FMDV (A, O, and Asia 1) have been isolated and characterized. Here, we retrieved all the nucleotide sequences of FMDVs isolated from mainland China, Hong Kong, and Taiwan (no data available for Macau) from the NCBI/GenBank database to understand the evolution and molecular epidemiology of FMDV. These genetic data were collected with the aim of (i) reviewing the history of FMD outbreaks; (ii) describing the current situation of FMD epidemics and their effective control; (iii) determining the genomic regions corresponding to important biological functions; and (iv) conducting phylogenetic analysis to detect potential recombination and selection events.

1 The history of FMD outbreaks in China (1958–1996)

The first reports of FMDV isolates were in 1958. A
serotype O isolate (O/Akesu/CHA/58) was collected from Akesu prefecture [11], a serotype A isolate (A/XJ/KT/CHA/58) was collected from Aketao county of the Xinjiang Uygur Autonomous Region [12], and a serotype Asia 1 isolate (Asia 1/YN/BS/CHA/58) was collected from Baoshan in Yunnan Province [13]. Since then, only a few minor FMD outbreaks (of serotype A) were detected in Gansu Province in 1962 and in the Inner Mongolia Autonomous Region in 1960 and 1964 (involving 2 infected premises (IPs)). The social, economic, and political problems in mainland China reduced the effectiveness of veterinary services up until the era of reform and opening-up. Around 20 years later, strain O/GD/China/86 strain was reportedly isolated from swine [14,15]. Hong Kong has reported the presence of serotypes O and Asia 1 from 1970–1996 and 1974–1980, respectively.

2 Current situation regarding FMD epidemics in Taiwan, Hong Kong, and mainland China (1997–2010)

2.1 Serotype O

Serotype O is the most prevalent and widely distributed serotype of FMDV globally, especially throughout the Middle East-South Asia (ME-SA) region [16]. Japan, Korea, Mongolia, Russia, the United Kingdom, France, and South Africa reported the presence of serotype O FMDV from 2000–2006, with all of these isolates belonging to the PanAsia group of strains [7,17–21]. According to the information provided by the Food and Agriculture Organization (FAO), the reoccurrence of FMDV serotype O in Japan, Korea, and Mongolia was confirmed in April 2010 (http://www.wrlfmd.org).

In 1997, an epizootic in Taiwan, a province of China, was caused by a serotype O FMDV. Disease was restricted to host pigs (family Suidea), and more than 4 million pigs had to be slaughtered [22–24]. FMD was endemic in Hong Kong between 1999 and 2004 [25–28]. In our survey, the presence of serotype O virus was reported in mainland China during a similar period (1999–2005) [29–33]. Epidemiological data and genetic characterization of circulating field viruses, as well as their relationships to vaccine strains that are employed in these areas, have been clarified. Efforts to control the movement of virus have been implemented by systematic vaccination. However, outbreaks of serotype O reoccurred in Taiwan in 2009 and in Hong Kong in May 2010 [34]. FMD outbreaks in mainland China have been prevalent. On 22 February 2010, the first confirmation of the disease was declared in Baiyun district in Guangdong Province. Up until late 2010, more than 18 IPs in 9 provinces had been detected (http://web.oie.int/wahis/public.php).

2.2 Serotype Asia 1

Members of this serotype are primarily restricted to Asia with recent incursions in the ME region, Korea (2007), Mongolia (2005), Russia (2005), Turkey, and Greece (2000) [35–38].

In 2005, FMDV of serotype Asia 1 was detected in cattle in Wuxi in Jiangsu Province. The disease spread rapidly and widely throughout mainland China, and a total of 46 IPs in 17 provinces reported outbreaks of FMDV serotype Asia 1 from 2005–2009 [39–42]. The presence of serotype Asia 1 virus was also reported in Hong Kong in March 2005 [43]. These outbreaks were gradually controlled.

2.3 Serotype A

Of the 7 serotypes, serotype A displays the highest antigenic diversity, and recombination in this serotype occurs more frequently than in other serotypes. However, the origin of the recombinant virus remains unclear [44,45]. Epidemiological investigations have revealed new outbreaks of FMDV serotype A in dairy cattle and deer in Korea since January 2010. These cases were confirmed by the isolation and identification of samples, clinical signs, positive results for antigen detection by an enzyme-linked immunosorbent assay, and nucleotide sequencing of the complete capsid protein VP1 coding region.

Since 1958, there have been no major outbreaks reported of this serotype in China. However, there is still no explanation for the emergence of an epidemic of FMDV serotype A in Hubei and Shanghai in early 2009. Phylogenetic analysis revealed that these isolates belonged to the South-East Asia (SEA) topotype. Subsequent outbreaks of FMD have spread to 10 IPs in 8 provinces (http://web.oie.int/wahis/public.php).

3 Molecular genetic characterization of FMDV VP1 protein, 3A protein, and pseudoknots

3.1 VP1

VP1 is the preferred region for comparison of FMD outbreak strains because of its significance for the analyses of serotype specificity, the dynamics of quasispecies evolution, the occurrence of antigenic heterogeneity, and the interactions of FMDV with cells [4,46,47]. In comparison with the VP1 sequence of serotype A, a deletion exists at nucleotide positions 427–432 of the VP1 sequence of serotype Asia 1 and a deletion/insertion exists at nucleotide positions 430–432/586–588 of the VP1 sequence of serotype A. Amino acid residues 133–158 (inclusive of VP1) form a G-H loop structure constituting important antigenic determinants, which protrude from the viral surface [48]. The tripeptide RGD motif within the G-H loop is a cellular recognition site which combines with integrins during the process of virus-receptor interactions. While sequences surrounding the RGD motif within the G-H loop vary, the RGD motif itself is highly conserved [49,50]. Storey et al.
[51] found that a second RGD motif in the VP1 capsid protein of SAT 1-type FMDV field isolates is not essential for attachment to susceptible cells. The coevolution of antigenicity and host cell tropism of FMDV suggests that the antigenic changes could be associated with alterations in cell receptor usage [52]. Glycosaminoglycan heparin sulfate (HS) has been reported as another receptor for a subtype of FMDV serotype O [53]. The rapid evolution of these antigenic sites in FMDV in nature hinders synthetic vaccines from providing efficient protection [54–56]. Phenotypic variability of viral RNA is strongly influenced by high mutation rates and quasispecies dynamics [57].

For comparison of the full and partial VP1 nucleotide sequences of viral isolates, a phylogenetic tree was constructed using the neighbor-joining algorithm [58]. The data revealed 113 isolates of the FMDV serotype O from China (7 isolates with partial VP1 sequences), grouping in the Cathay, PanAsia, and SEA topotypes. The porcineophilic lineage strains (of the Cathay topotype, including the HKN70, HKN90, and TAW97 genotypes) of FMDV were the pathogens responsible for the pandemics in Hong Kong and Taiwan during the 1990s, and the recent outbreaks in the mainland and Hong Kong (2010) were caused by the SEA topotype (Mya98 strain) of FMDV serotype O [59]. A total of 35 isolates of FMDV serotype Asia 1 from China (two isolates with partial VP1 sequences) were subdivided into the CHA58, Group II, HKN74 and Group IV (SEA topotype), and Group V genotypes (Figure 1). Amino acid sequence alignments have shown that the RGD motif is lacking in some virus isolates (the tripeptide SGD motif, O/Akesu/CHA/58) and certain variants use an RGD-independent infection mechanism that does not require binding to the surface HS. This suggests the potential use of at least 3 alternative receptors for entry of FMDV into target cells [60]. FMDV mutability and the simultaneous existence of several motifs (RGD, RDD, and RSD) within FMDV populations in vitro may contribute to memory in viral quasispecies [41] (partly unpublished data).

Phylogenetic statistical analysis clearly showed that groups of viruses share common geographical characteristics and epidemic histories. However, these data do not provide information regarding the origin of viruses and the sources of occasional global pandemics. The phylogenetic tree revealed that certain isolates may play a significant role in the genetic evolutionary dynamics of FMDV. Isolate Asia 1/YNYML/CHA/2000 (CHA58 genotype) collected from unvaccinated cattle, indicates that epidemiological investigation is responsible for not only new outbreaks caused by novel FMDV strains, but also the re-emergence of new cases caused by “old” virus isolates.

3.2 3A

3A protein is likely to play a role in viral virulence and the determination of host range. Changes in protein 3A have been reported in several virus isolates belonging to serotypes O and C, which have become attenuated in cattle but remain pathogenic in pigs. Molecular characterization of these viruses revealed that amino acid deletions (10-, 11-, 19-, or 20-codons) and series of substitutions occurred in the C-terminal half of the 3A coding region [17,23,61]. Deletions and mutations in protein 3A appear to be responsible for this particular phenotype, and they affect RNA synthesis in keratinocytes of swine origin less than those of bovine origin [17].

In a comparison of the 3A nucleotide sequences of 80 FMDV isolates (including 47 isolates collected in China), an unrooted neighbor-joining tree showed coevolution in accordance with the sequence alignment based on the nucleotides in the VP1 region (Figure 2). There were 10 amino acid deletions of codons at positions 93–102 in the porcinephilic lineage (Figure 2). In addition, isolate Asia 1/HKN/CHA/2005 was found to have the highest level of similarity with PanAsia strains in the 3A region (95.1% on average), indicating a closer relationship between Asia 1/HKN/CHA/2005 and PanAsia strains. The series of amino acid substitutions originated in the porcinephilic lineage and resulted in fitness gain or loss (from quantitative variation to qualitative alteration), which may affect cell tropism and host range during FMDV evolution. In some cases, the Q44R mutation in the 3A protein was associated with the adaptation of FMDV to guinea pigs [62].

Fifteen amino acid deletions (131–145) of OMIII (artificially attenuated from strain O/Akesu/CHA/58) were displayed in the 3A protein (Figure 2). Another strain of serotype O isolated from bovine and identified during the 35th passage in swine cells, contained 18 amino acid deletions (85–102) in the 3A region (unpublished data). Baranowski et al. [60,63,64] documented that the FMDV clone C-S8c1, a virus recovered after 100 persistent passages of carrier BHK-21 cells, resulted in increased virulence of the BHK-21 cells and altered cell tropism. Several amino acid substitutions were identified in this clone around the G-H loop. These data showed that large population passages of FMDV may lead to host cell tropism alterations [4]. Interestingly, Oem et al. [65] also showed that a Korean 2002 isolate (PanAsia strain, O/AS/SKR/2002), which contained an intact 3A coding region, caused an infection that was highly virulent and contagious in pigs, but limited in cattle. Analysis of this virus found that mutations in other regions correlated with changes in the VP1 and 3A coding regions of the FMDV genome, and may indicate alterations in the host range.

Furthermore, 3A is a transmembrane protein that contains hydrophobic sequences (61–76 aa) and the expression of FMDV 3A protein caused a disruption of the Golgi apparatus [66]. Structural prediction of the 3A protein revealed a conserved N-terminal helix (1–92 aa) and a C-terminal random-coil conformation (93–153 aa) similar to...
Figure 1  Phylogenetic tree of the VP1 nucleotide sequences of FMDV generated using the MegAlign software. The 197 FMDV isolates comprised 127, 49, and 21 isolates of serotype O, Asia 1 and A, respectively. * The partial VP1 sequence data of these isolates. n Number of isolates in each group.
Figure 2 Unrooted neighbor-joining tree generated from the nucleotide sequences of the 3A region and alignment of the amino acid sequences of the 3A protein of FMDV isolates (from codon positions 89 to 108, and 128 to 147, respectively).
phosphoprotein enriched in astrocytes of 15 kD (PEA-15), indicating that FMDV 3A is an “on-off switch” required for manipulating integrin activation in a Ras-dependent manner (Zhang et al., unpublished data).

3.3 Pseudoknots (PKs)

PKs have been predicted to be involved in conserved structures that are highly tolerant to changes at the 3′ termini of the poly(C) tract of FMDV genomes. PKs are believed to play a crucial role in the RNA replication cycle and the origin of polyprotein synthesis [67,68]. There is some evidence of nucleotide sequence deletions in this region in serotypes A, C, and O [69–71]. These data raise interesting possibilities regarding the functional constraints for sequence variability with biological implications (virulence, alteration of host range, etc.) and the population dynamics of FMDV evolution, depending on the potential structures of PKs analyzed in the present study.

An unrooted neighbor-joining tree was determined based on an alignment of the complete PK sequences (Figure 3). It was highly similar to phylogenetic trees generated from the nucleotide sequences of the VP1 and 3A regions, indicating shared evolutionary pathways under artificial conditions, and/or physical environments. Sequence analysis revealed a 47- and 42-nucleotide deletion in the PKs of OMIII (132

Figure 3 Unrooted neighbor-joining tree of the pseudoknot (PK) nucleotide sequences of FMDV isolates generated using the MEGALIGN software. * The partial PK sequence data of these isolates.
nucleotides) and the porcineophilic lineage strains (175/176 nucleotides), respectively, compared with isolates O/Akesu/CHA/58 (179 nucleotides) and the PanAsia strains (217/218 nucleotides). Nucleotide deletions were also detected in the PKs of serotypes A, Asia 1, C, and SAT 1–3, which may provide insight into genetic relationships between different lineages, and could be used as genetic markers [71].

The nucleotide deletions in these strains resulted in the loss of one or two of the three or four conserved PK repeats at the 5′-end. Remarkably, these changes may strongly hinder the process of host factor recruitment. This in turn allows the viral RNA to efficiently multiply during a relatively short infection cycle, and may affect pathogenesis and host cell tropism of FMDV. In retrospect, the “cloverleaf” structure at the non-coding region of a subgroup of picornaviruses, which include polioviruses, coxsackie B3 viruses, enteroviruses, and human rhinoviruses, implies the direct influence on the evolutionary dynamics of the 3′-end of PKs involved in the replication of FMDV as a cis-acting element [68,72,73].

4 Recombination analysis of genetic variation, genomic mosaicism, and extension

Complex genetic variation mechanisms are responsible for the existence of different serotypes of FMDV, resulting in altered antigenic properties and modified virulence. Phylogenetic trees generated from cross-over regions of individual isolates among and within serotypes revealed intra- and/or inter-serotype recombination (A, O, C, and Asia 1) and a novel SAT lineage (which probably plays an important role in FMDV evolution and convergent evolution) will may affect both recombination detection and phylogenetic estimates [38,40,44,45,71,74–77]. Diverse breakpoint patterns occur mainly in the non-structural protein coding regions, and occasionally in the capsid coding regions.

A recent study proposed that a mosaic structure with a partial 2C fragment of Asia 1/JS/CHA/2005 was transferred from FMDV isolate Asia 1/HKN/CHA/2005 [38]. Recombination results in genetic exchange among parental viruses to generate progeny strains. This process could also explain the nonstructural protein coding region extension of FMDV genomes with degraded RdRp activity. Similarity plots and bootscanning analysis were used to assess the authentic relationships of Asia 1/HKN/CHA/05 with several reference strains. We detected the occurrence of putative gene recombination between the O/Tibet/CHA/1/99 PanAsia strain (non-coding and nonstructural protein coding regions) and the Asia 1/IND/321/01 strains (capsid coding regions). These strains recombined to produce the Asia 1/HKN/CHA/05 strain (Figure 4). These results are analogous to the findings of previous studies, although the percentage identities of the VP1 nucleotide sequences between Asia 1/HKN/CHA/05 and Asia 1/IND/321/01, Asia 1/IND/491/97 are 89.4% and 91.2%, respectively [13,40]. This level of divergence with their parental genomes can be explained by the mutation rates per genome per replication cycle [75,78,79]. The recombination among natural FMDV populations suggests that FMDV genome diversity may exist extensively in the field [71]. Mixed infection (inter-serotype and intra-serotype) is common and positive selection occurs under natural environment, which limits the opportunity for the accumulation of genetic variation and multiple infections by different genotypes [80].

5 Selection detection of the G-H loop in the VP1 surface capsid protein

The capsid of FMDV presents many neutralization epitopes that cluster in several exposed regions. The G-H loop of VP1, one of the immunodominant sites, appears as a loosely connected element that may undergo hinge movements, and may adopt preferred orientations depending on the specific virus strain. The G-H loop exhibits conserved structural features. Severe restrictions on sequence variation in the G-H loop occur because of the dual involvement of some
residues, including the tripeptide RGD motif and some neighboring residues, in both antibody reactivity and cell attachment [81–84].

Phylogenetic analysis of genetic diversity and adaptive molecular evolution was also estimated under selection detection at the amino acid level for the G-H loop of serotypes O and Asia 1 (Table 1). In serotype O, there were 4 residues under selection within the Cathay topotype, and residue 135 was under selection within the natural bovine-isolated Mya98 strain. In the porcinophilic lineage, 5 residues were under stabilizing selection and four residues were under destabilizing selection within the HKN70 strain. Positive-stabilizing selection within the HKN90 and TAW97 strains occurred at 6 positions and positive-destabilizing selection occurred at 3 positions in this region. Positive selection at specific amino acid sites within the G-H loop of VP1 in serotype O isolates has been proposed to be critical in the formation of neutralizable antigenic sites [77,85–87]. Analysis of sequence of serotype Asia 1 strains resulted in the detection of 3 residues under stabilizing selection within Group V, 8 residues under stabilizing selection within Group II, 7 residues under stabilizing selection, and residue 140 under destabilizing selection (140Thr in Group IV and 140Pro in HKN74 strains) within the SEA topotype. Residue 136 is one of the most highly conserved amino acids in the G-H loop (with the exception of O-TW-256-2001). Residues 144, 149, and 150 are distinguishable among different serotypes, and residues 154, 155 (with the exception of O-TW-255-2000), and 158 are distinct in serotype O strains compared with strains of the two other serotypes. These results may contribute to our understanding of the affinity of antibody recognition and the effectiveness of synthetic peptide vaccines.

### 6 Concluding remarks

In epidemiological surveys of FMD, molecular characterization

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**Table 1** Selection detection of amino acid residues within the G-H loop (133–158)\(^a\) of capsid protein VP1

| Genotypes | PanAsia | SEA | Cathay | Serotype Asia 1 |
|-----------|---------|-----|--------|----------------|
|           | Mya98   | CHN58 | HKN70 | HKN90 | TAW97 | Group V | Group II | Group IV | HKN74 | CHA58 |
| 133       | N       | D    | N      | S      | S      | S      | K       | M     | K      | N/T1 | K    |
| 134       | C       | C    | Y     | C      | S      | N    | T      | T     | T      | T    | T    |
| 135       | K       | R    | K     | K      | K      | K      | T      | T     | A      | A    | T    |
| 136       | Y       | Y    | Y     | Y      | Y      | Y      | Y      | Y     | Y      | Y    | Y    |
| 137       | G       | D    | A    | S      | N      | S      | G      | G     | G      | G    | G    |
| 138       | E       | K    | E    | G      | N      | D    | D      | D      | N      | E    | G    |
| 139       | S       | G    | G    | S      | N      | A    | T      | T     | E      | T    | E    |
| 140       | P       | P    | S    | N      | R    | H    | S      | S      | P/T1   | T    | P    |
| 141       | V       | L    | V    | V      | M/A1  | T    | T      | S      | P/T1   | E    | T    |
| 142       | T       | A    | T    | S      | S    | G    | T      | N    | S      | R    | R    | R    |
| 143       | N       | N    | N    | K/e     | N    | N    | T    | N      | –      | –    | –    | –    |
| 144       | V       | A/L3 | V    | V      | L2    | V    | V      | V      | –      | –    | –    | –    |
| 145       | R       | R    | R    | R      | R      | R      | R      | R      | R      | R    | R    |
| 146       | G       | G    | G    | G      | G      | G      | G      | G      | G      | G    | G    |
| 147       | D       | D    | D    | D      | D      | D      | D      | D      | D      | D    | D    |
| 148       | L       | L    | L    | L      | L      | L      | M    | L    | L      | F    | P    |
| 149       | Q       | Q    | Q    | Q      | R      | Q    | Q      | A      | A      | A    | A    |
| 150       | V       | V    | V    | V      | V      | V      | A      | A    | A      | A    | A    |
| 151       | L       | L    | L    | L      | L      | L      | P    | L    | L      | L    | L    |
| 152       | A       | T    | A    | A      | D    | T    | A    | A      | V    | A    | A    |
| 153       | Q       | P    | Q    | Q      | Q      | Q      | Q      | R    | H    | Q    | Q    |
| 154       | K       | K    | K    | K      | K      | K      | R      | R    | R      | R    | R    |
| 155       | A       | A    | A    | A      | T    | A    | V    | L    | V    | V    | L    |
| 156       | A       | A    | A    | A      | A      | A      | E    | E      | E    | N    | S/D2 |
| 157       | R       | R    | R    | R      | R      | R      | N      | G    | R      | R    | G    |
| 158       | T       | P    | P    | P      | A    | A    | T    | T    | T      | A    | A    |

\(a\) Residue numbers correspond to serotype O; the most frequent amino acid for each strain is shown in the top row; specific residues for serotype O are shown in italics; residues of tripeptide motif within the G-H loop are shown in bold; –, residue deletion.
of FMDV genetic diversity provided insight into the relationships between different field isolates. Despite significant advances in our understanding of viral pathogenesis and the development of new antiviral strategies in the last two decades, FMDV remains a major threat to animal husbandry in China. The control of FMD depends on: (i) strategic vaccine reserves, which can confer rapid and protective immunity in healthy and susceptible species in an outbreak (e.g., the North American Vaccine Bank and the European Union Vaccine Bank) [56]; (ii) the establishment of immune bands around national border lines to interrupt airborne transmission. FMD can be transmitted by airborne viruses many kilometers away from the virus source. It has been shown that aerosol exposure results in the typical clinical signs of FMD or persistent subclinical infections of FMDV in animals [88,89]. This in turn may lead to genetic drift in less fit genotypes of the virus [37]; (iii) understanding the global epidemiology of FMDV, especially in neighboring countries and areas, and implicating control measures such as transport restrictions of susceptible animals and the quarantine of animal products [90,91]; and (iv) introducing strict biosafety measures and the prudent application of innovative technologies to prevent the viruses from escaping from research laboratories [9,92]. These findings may prove beneficial in formulating control measures against FMD by the Chinese government.

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