Whole Genome Sequencing of A Candidate Strain for FMDV Vaccine: Genomic Structure and Genetic Variation

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Abstract The complete genome of foot-and-mouth disease virus (FMDV) vaccine candidate strain O/YM/YN/2000 was sequenced and analyzed. Seven overlapping cDNA fragment which covered the O/YM/YN/2000 genome were amplified by RT-PCR and sequenced. The results showed that the complete genome of the strain O/YM/YN/2000 has 8 133 nucleotides (nt) in length including a 1 053 nt 5′-untranslated region (UTR) (including 18 nt poly C), a 6 969 nt open reading frame (ORF), a 93 nt 3′-UTR and at least 18 nt poly A tail. The homologous comparison and phylogenetic analysis of the nucleotide and deduced amino acid (aa) sequences among O/YM/YN/2000 and other FMDV strains available deposited in GenBank were performed. Phylogenetic analysis based on the complete genome and VP1 revealed that that the strain O/YM/YN/2000 is clustered in the FMDV serotype O cathay topotype. Fragment deletions mutation in O/YM/YN/2000 were revealed, deletions involving 4 nt in the S fragment of 5′-UTR, 43 nt which possibly contributed to the loss of pseudo-knot domain II of 5′-UTR and 10 aa residues in 3A protein. The results in this research might provide some information for screening FMDV vaccine candidate strain.

Keywords FMDV (foot-and-mouth disease virus); Vaccine; Genome sequence; Genomic structure and Genetic variation

Background Foot-and-mouth disease (Aphtae epizooticae), belongs to Picornaviridae family of Aphthovirus genus, is an infectious and fatal viral disease that affects cloven-hoofed animals, including domestic and wild animals, which is a severe plague for animal farming due to its highly infectious and easy spread. Foot-and-mouth disease virus (FMDV) causes a high fever for two or three days, followed by blisters inside the mouth and on the feet that may rupture and cause lameness. FMDV has a positive-sense single-stranded RNA genome and exists in seven immunologically distinct serotypes, i.e. Euroasiatic serotypes A, O, C, Asia 1, South African territories SAT 1–3) and multiple subtypes (Brown, 2003). Infection with one of serotype virus does not confer immunologically protection against other serotypes and antigenic variation within serotypes might come out outbreak strains that vaccines must be carefully matched to ensure efficacy (Parida, 2009). The FMDV genome, which has about 8.0 kb in size, contains a 5′-untranslated region (5′-UTR), a single open reading frame (ORF) and a 3′-untranslated region (3′-UTR) (Mason et al., 2003). The 5′-UTR consists of a short fragment (S fragment), a poly (C) tract, a long fragment with three or four tandem repeat pseudoknots (PKs) and an internal ribosome entry site (IRES) (Belsham, 2005; Mason et al., 2003). The FMDV ORF encodes a polypeptide that is cleaved to form mature polypeptide products, which includes four structural proteins (VP1, VP2, VP3 and VP4) and eight non-structural proteins (2B, 2C, 3A, 3B, 3D, Lpro, 2A and 3Cpro) (Belsham, 2005; Carrillo et al., 2005; Mason et al., 2003). The 3′-UTR is about 90 nt in length with a 35–100 nt poly (A) tail (Belsham, 2005; Carrillo et al., 2005).

Molecular epidemiological studies on FMDV were
usually focus on sequencing the part or all of the genome region coding for the outer capsid protein VP1. If the isolate with more than 85% nt sequence identity has been placed within group or topotype, which tends to be restricted in their geographic distribution (Knowles and Samuel, 2003). FMDV type O is the worldwide pandemic virus serotype, which can be grouped into eight topotypes that is 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 15% nucleotide differences (Samuel and Knowles, 2001). In addition, the FMDVs still exist in different evolutionary sublineage within the topotypes or groups.

Recently, methodologies for FMDV vaccine strain selection mainly rely on serological approaches (Paton et al., 2005). The determination of FMDV nucleotide sequences and phylogenetic analysis is the supplement approaches for selecting vaccine strain to use in FMD control program (Reeve et al., 2010). In 1997, a devastating outbreak of foot-and-mouth disease, with atypical virulence which produced high morbidity and mortality in swine but did not affect cattle, occurred in Taiwan (Dunn and Donaldson, 1997). We isolated a FMDV strain with similar biological characteristics as Tanwan’s isolates in Monitoring Station of Border Region of Yunnan Province. Previously, the biological characteristics and immunogenicity of this isolate were studied. In this paper, we presented the full-length genome sequence of this isolate and analyzed its genetic characteristics in order to provide a ground for selecting the vaccine candidate strain in FMD prevention and control.

1 Results
1.1 Amplifying the whole genome of O/YM/YN/2000 isolate
In order to sequence whole genome of the O/YM/YN/2000 isolate, seven primer sets were used for amplifying the whole genome and then cDNA was synthesized with the 6 nt random primer. Seven fragments (S1, S2, F2, F3, Fa, F4b and F5) spanning whole O/YM/YN/2000 genome were generated by PCR. The expected sizes of 374 bp, 637 bp, 1 107 bp 2 169 bp, 2 454 bp, 1 571 bp and 1 533 bp in length were synthesized in this research (Figure 1).

![Figure 1 The fragments amplified from FMDV strain O/YM/YN/2000 by RT-PCR](image)

Note: M1: DNA Marker DL2000; M2: 500 bp DNA Marker

1.2 Sequencing whole genome of O/YM/YN/2000
The whole genome of O/YM/YN/2000 strain is 8 133 nt in length, which includes a 1 053 nt 5´-UTR (including 18 nt continuous poly C tract), a 6 969 nt length open reading frame (ORF) encoding a polyprotein of 2 323 amino acids (aa) residues or 2 294 aa due to two alternative initiation sites separated by 84 nt, a 93 nt 3´-UTR and 18 nt poly A tail. Table 1 presented the genomic organization of O/YM/YN/2000 (nucleotides and deduced amino acids). The genome sequence has been deposited in to the Genbank database of the NCBI with Accession Number HQ412603.

1.3 Genomic comparative analysis between O/YM/YN/2000 with other known strains
The nucleotide and deduced aa sequences of each region (5´-UTR, L, VP4, VP2, VP3, VP1, 2A, 2B, 2C, 3A, 3B, 3C, 3D and 3´-UTR) of O/YM/YN/2000 and other FMDV reference strains were compared in this research (Table 1). Regarding to coding region, the sequence of non-structural proteins is much more conserved than that of the structural proteins in the FMDV genome. Among non-structural protein coding regions, 2A (93.7%~100%), 2B (90.2%~100%) and 3D (94.2%~99.4%) are the most highly conserved region. While in the structural protein regions, VP1 gene is highly variation (52.1%~93.9%) identity in nucleotide sequences), VP4 shows the lowest sequence identity (91.6%~99.2%). In the non-structural protein coding regions, L (61.2%~95.3% identity in aa sequence) and 3A (68.2%~96.6%) exist more diversity. Among all the compared regions, 5´-UTR, VP1 and 3A would be the lowest conservative.
Table 1 The genomic structure of O/YM/YN/2000 (nucleotides and amino acids) and sequence similarities compared with other FMDV reference strains

<table>
<thead>
<tr>
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<th>Nucleotides</th>
<th>Amino acids</th>
<th>Nucleotide similarity (%)</th>
<th>Amino acid similarity (%)</th>
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<td></td>
<td>Minimum</td>
<td>Maximum</td>
<td>Minimum</td>
<td>Maximum</td>
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<tr>
<td>5’-UTR</td>
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<td>L</td>
<td>603</td>
<td>201</td>
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<tr>
<td>VP4</td>
<td>255</td>
<td>85</td>
<td>80.2</td>
<td>95.4</td>
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<tr>
<td>VP2</td>
<td>654</td>
<td>218</td>
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<td>92.6</td>
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<td>VP3</td>
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<tr>
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<td>633</td>
<td>211</td>
<td>52.1</td>
<td>93.9</td>
</tr>
<tr>
<td>2A</td>
<td>54</td>
<td>18</td>
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<tr>
<td>2B</td>
<td>462</td>
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<td>2C</td>
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<tr>
<td>3’-UTR</td>
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<td>74.7</td>
<td>90.4</td>
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</tbody>
</table>

1.4 Phylogenetic analysis

Sequence analysis of O/YM/YN/2000 with the references of 19 other reported FMDV genomes suggested that the O/YM/YN/2000 strain should be of serotype O. Figure 2 showed a neighboring-joining (NJ) tree constructed based on the sequence alignment of employed 20 genomes, which were distinctly divided into 7 serotypes. O/YM/YN/2000 was tightly clustered in the O serotype and closely linked to O/HKN/2002, O/Taiwan/97, O/Chu-pei, O/LZ and O/ES/2001, with close genetic distances.

Moreover, based on the study of molecular epidemiology using the nucleotide sequence of the known VP1 gene, the VP1 gene of O/YM/YN/2000 represented the eight topotypes of serotype O compared with other 40 reference strains. The phylogenetic analysis indicated that O/YM/YN/2000 should belong to the same branch as Cathay topotype (Figure 3), and its typical biological characteristic of this lineage virus strain was highly pathogenic in swine.

1.5 Comparison of 5’-UTR between O/YM/YN/2000 and reference strains

The 5’-UTR of O/YM/YN/2000 has 1036 nt in length (excluding the poly C), containing a short segment, S-fragment, between 5’-end of genome and poly C tract which is 364 nt in length. A 4 nt deletion in S-fragment of O/YM/YN/2000 is evident (Figure 4A). The predicted RNA secondary structure of the S fragment would be a single long stem-loop structure (not shown figure). Although the deletion has no any evidence to influence on the predicted secondary structures, this deletion might change some biological characteristics of the virus, because the S-fragment presumably involved in replication. The S-fragment and poly(C) tract are followed by a segment of RNA of 672 nt in length that can form a number of highly conserved secondary structures. These structures include tandem repeat pseudoknots (PKs), the cis-acting replication element (cre), and the Internal Ribosome Entry Site(IRES). Most FMDV strains have four PK structures, PK I, II, III and IV sequentially, located in the function unknown region (FUR) at the 3’-end of poly (C) tract. Compared with the reference strains, a 43 nt consecutive deletion occurring in the 5’-UTR of O/YM/YN/2000 results in lost of one of PK structure, and this deletion exists in 5 strains including strains of the Cathay and Middle-East South Asia (ME-SA) topotype (Figure 4B). It is unknown whether or not the function of missing PK II structure does change the tropism of virus.

1.6 Comparison of VP1 and 3A protein between O/YM/YN/2000 and reference strains

Comparison of structural protein VP1 amino acid sequences between O/YM/YN/2000 and reference strains were conducted in this research. The results of three major antigenic sites, 40~60, 133~160, and
200–211 were presented in Figure 5, in which 40–60 and 133–160 aa segments have a significant variation, and C-terminal linear epitope 200–211 aa of VP1 protein showed much more conservative. The 133–160 aa of VP1 would facilitate GH loop structure, which contains the highly conserved arginine-glycine-aspartate (RGD) cell adhesion sites. Two leucine residues located at positions +1 and +4 downstream of the RGD were highly conserved. In addition to the exquisitely specific RGD triplet, the residue at position +2 were the only critical and specific determinant within the loop in promoting cell recognition of a viral ligand (Mateu et al., 1996).

Comparison of non-structural protein 3A showed that a 10 aa deletion (position 93–102) in the 3A protein is observed (Figure 6). A similar deletion exists in the virus strain lineages of Cathay topotype (Taiwan/97, O Chu-peii, O/HKN/2002 and O/LZ strain). The location of this change is different from the portion of 3A containing deletions in the chicken embryo-adapted FMDVs (Giraudo et al., 1990) and Asian isolates of a type O FMDV with the high virulence for swine (Beard and Mason, 2000; Knowles et al., 2001; Núñez et al., 2001), which might be an alternative mechanism of host spectrum alteration.

2 Discussions
Sequencing and analysis of FMDV isolates for studying virus structure and function would be one of the very important way, because sequence differences
between the FMDV genomes are often manifest in differences of biology and pathogenicity. In this paper, we determined and analyzed whole genome sequence of O/YM/YN/2000 strain, those data, together with the previous research information about biological and immunological characteristics of the tested isolate, might provide a scientific basic knowledge for selecting swine FMD serotype O vaccine candidate to
The 5’-UTR of FMDV S fragment were known to fold a stem-loop structure (Clarke et al., 1987), but its function is not yet clear. The poliovirus, sharing the same family of picornavirus with the FMDVs, its 5’-terminal of RNA fold into a cloverleaf RNA structure, which has been shown to be involved in genome replication and RNA stability, can produce the interactions with the viral and cellular protein (Parsley et al., 1997). Those results suggested that the S segment of FMDV 5’-UTR might be involved in genome replication and maintaining viral RNA stability in the infected cells. Other speculated that the virus S segment might affect pathogenicity of virus, but there is no evidence to be confirmed. In the S segment of O/YM/YN/2000, 5’-UTR 4 nt deletion exists there (Figure 4A), but compared to reference strains there are no deletion in the S segment. The 4 nt deletion would be a specific feature of O/YM/YN/2000, and the deletion might be associated with the biological characteristics of strain, it would be worth studying in future. In addition, Most FMDV strains have four of PK structures, the 43 nt deletion located in the region containing in PK structures of O/YM/YN/2000 5’-UTR might cause the loss of PK II domain (Figure 4B). This deletion presents in the seven serotype of FMDVs, it is assumed that the deletions in the PK regions are common in the evolution of FMDV, and loss of one or two PKs has been reported (Escarmis et al., 1995; Feng et al., 2004). The function of PKs is still not clear, but it is assumed that the PK structure, combining with the S segment and the poly (C) tract, might play a role in genome replication (Mason et al., 2003). Qian Fang et al proposed that the loss of PK II domain of HKN/2002 might be involved the variation in the
virulence of virus and host tropism (Feng et al., 2004). So far, few PKs with virulence-related research are reported.

FMDV mutations mostly happened in the capsid protein coding regions, of which the VP1 would be the largest variation region, and their two antigenic sites located on 42–60 aa (B-C loop) and 133–158 aa (G-H loop) are a highly variable region. The antigenic variation created by sequence differences resulted in lack of immunologic cross-reactivity among outbreak strains using sera from vaccinated animals (Mattion et al., 2009). The binding sites for RGD cell receptor located the G-H loop of VP1 gene of O/YM/YN/2000 did not change any more (Figure 5), and phylogenetic analysis displayed the virus cluster with the Cathay topotype (Figure 3). Therefore, the O/YM/YN/2000 would be selected to be as a potential promising vaccine candidate strain, which provided the genetic information about the epitopes to play dominant roles in vaccine-induced protection. It was recognized that high antigenic variation in FMDV causes a major problem in selection of vaccine strain. Comparing sequence data generated from FMDV might be helpful to rapidly select candidate vaccine strain for better control the disease (Mattion et al., 2009; Parida, 2009). However, there is extensive antigenic variations within FMDV, the changes might be limited to very specific regions of the viral surface. The antigenicity of the tested vaccine candidate strain could not be predicted only based on the strains’ topotype or genotype but it would be enough for only selecting the vaccine strain using these genetic information. Therefore, vaccine strain selection might mainly rely on serological and immunological approaches, combining with the virus genetic analysis information. Previously, we carried out the antigenic relationship values (r values) between O/YM/YN/2000 (belongs to the Cathay topotype) and two other topotype (ME-SA and SEA), FMDV type O vaccine candidate strains, the serological cross-protection tests showed that the antigenicity of O/YM/YN/2000 can cover these two topotype isolates (r<0.3) (data not shown). So, to select the vaccine candidate strain, particular in selecting a broad spectrum of antigenic viruses, the immunogenicity and production performance of candidate strains should be considered to be known (Mattion et al., 2009).

In conclusions, O/YM/YN/2000, potentially as a FMDV vaccine candidate strain serotype O, belongs to the Cathay topotype. A 4 nt deletion in S fragment and 43 nt in FUR exist in the 5’–UTR of the O/YM/YN/2000. The arginine-glycine-aspartate (RGD) sequence located in the G-H loop of the VP1 protein might involved in binding of FMDV to cellular receptor. A 10–aa deletion in the 3A nonstructural protein was found in the O/YM/YN/2000 strain. Those genetic information of the O/YM/YN/2000, combining the previous studies on serology and pathology, might be helpful for identifying vaccine candidate strain, although its antigenicity and immunogenicity did need to be further clear.

3 Materials and Methods

3.1 Virus isolate
The O/YM/YN/2000 strain was isolated from the animal disease monitoring station of border region of Yunnan Province and isolate was passaged by baby hamster kidney (BHK–21) monolayer cell culture.

3.2 RT-PCR and Sequencing
Viral stock was propagated as previously described approach (Xin et al., 2009). Virus RNA was extracted from virus stock using the Viral RNA Extract Kit (Bio.Basic.Inc.) according to the manufacture’s instructions. Reverse transcription was carried out using 6 random primes and Superscript II reverse transcriptase (Invitrogen). Seven cDNA fragments covering the entire FMDV genome were amplified by PCR using seven primer sets (Figure 7). The primers showed in Table 2. PCR amplifications with Pyrobest

Figure 7 Strategies for amplifying the whole genome of FMDV O/YM/YN/2000 strain by RT-PCR
Molecular Pathogens

Table 2 Primer pairs used in PCR for amplifying whole O/YM/YN/2000 genome

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Name</th>
<th>Sequence (5´-3´)</th>
<th>Position a</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>F1</td>
<td>TGGAAAGGGGGGGCTAGGTCT</td>
<td>1~22</td>
</tr>
<tr>
<td></td>
<td>Rc</td>
<td>GGGGGGGGGGGTGAA</td>
<td>361~374</td>
</tr>
<tr>
<td>S2</td>
<td>F-ployC</td>
<td>CCCCCCCCCCTAARGYYYTACGWC</td>
<td>374~408</td>
</tr>
<tr>
<td></td>
<td>IRES4</td>
<td>CATTACGCGCTAGAAGCTT</td>
<td>991~1011</td>
</tr>
<tr>
<td>F2</td>
<td>F916</td>
<td>GACTGGGTGTTGAGGTCG</td>
<td>1974~1994</td>
</tr>
<tr>
<td>F3</td>
<td>OF3</td>
<td>CCACCCCTCTCGAGGACCCG</td>
<td>1931~1950</td>
</tr>
<tr>
<td></td>
<td>2BR</td>
<td>AGCTTGTTACCGGTTTGGC</td>
<td>4081~4100</td>
</tr>
<tr>
<td>F4a</td>
<td>2BF</td>
<td>CAGATGCGAGGAGCAGTC</td>
<td>3970~3989</td>
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<tr>
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<td>R6434</td>
<td>AGAGGCCAGGATGTTGC</td>
<td>6046~6424</td>
</tr>
<tr>
<td>F4b</td>
<td>F5404</td>
<td>GAAAGGGCACCAGGACGC</td>
<td>5373~5393</td>
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<tr>
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</tr>
<tr>
<td>F5</td>
<td>OF5</td>
<td>GGGTTGATCGTCGACACGAG</td>
<td>6610~6632</td>
</tr>
<tr>
<td></td>
<td>R-Not I dT18</td>
<td>GGGGGCGGCCGCGGT</td>
<td>Poly A</td>
</tr>
</tbody>
</table>

Note: a: Nucleotide positions corresponds to the nucleotide sequence of the FMDV O/YM/YN/2000

Pfu polymerase (Takara, Dalian) were performed according to the manufacturer’s protocol. For PCR amplification, PCR reaction mixtures was followed by 1 cycle for pre-denaturing 5 min at 95 °C, 30 cycles for amplification reaction at 94 °C for 30 sec, 58 °C (for S1 and S2 fragment) or 52 °C (for F2, F3, F4a, F4b and F5 fragment) for 30 sec, 72 °C for 1 min (for S1 and S2) or 3 min (for F2, F3, F4a, F4b and F5), and 1 cycle for final extension at 72 °C for 10 min. The PCR products were purified from agarose gel electrophoresis and sequenced directly by ABI-PRIS MTM 377XL DNA Sequencer in two companies, Sangon Biotech (Shanghai) and Taihe Biotech (Beijing).

3.3 Sequence and phylogenetic analysis
FMDV reference sequences were online acquired from the GenBank database of National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). The sequence data were assembled using the program Assemble (Vector NTI 8.0 suite, InforMax, North Bethesda, MD). Multiple sequence alignments were performed by ClustalX multiple sequence alignment program, version 1.83 (Thompson et al., 1997). The phylogenetic tree was constructed by neighbor-joining method in MEGA version 3.1 (Kumar et al., 2004) and the reliability of the branching orders was evaluated using the bootstrap test (n=1000).

Author contributions
XAG developed the laboratory protocols, sequenced the genomes for the FMDV sequencing project, conducted the phylogenetic analysis and wrote the paper. LE and YYQ collected the clinical isolates, selected the subset for sequencing, MHS prepared the viral RNA. ZMW and SCH performed laboratory assurance and animal experimental test. LDF managed development of bioinformatics software for assembly. LHC and XAG contributed to overall project management.

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