Cis-acting Replication Element Variation of the Foot-and-mouth Disease Virus is Associated with the Determination of Host Susceptibility

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The foot-and-mouth disease virus (FMDV), a member of the Aphthovirus genus in the Picornaviridae family, affects wild and domesticated ruminants and pigs. During replication of the FMDV RNA (ribonucleic acid) genome, FMDV-encoding RNA polymerase 3D acts in a highly location-specific manner. This suggests that specific RNA structures recognized by 3D polymerase within non-coding regions of the FMDV genome assist with binding during replication. One such region is the cis-acting replication element (CRE), which functions as a template for RNA replication. The FMDV CRE adopts a stem-loop conformation with an extended duplex stem, supporting a novel 15-17 nucleotide loop that derives stability from base-stacking interactions, with the exact RNA nucleotide sequence of the CRE producing different RNA secondary structures. Here, we show that CRE sequences of FMDVs isolated in Korea from 2010 to 2017 exhibit A and O genotypes. Interestingly, variations in the RNA secondary structure of the Korean FMDVs are consistent with the phylogenetic relationships between these viruses and reveal the specificity of FMDV infections for particular host species. Therefore, we conclude that each genetic clade of Korean FMDV is characterized by a unique functional CRE and that the evolutionary success of new genetic lineages may be associated with the invention of a novel CRE motif. Therefore, we propose that the specific RNA structure of a CRE is an additional criterion for FMDV classification dependent on the host species. These findings will help correctly analyze CRE sequences and indicate the specificity of host species for future FMDV epidemics.

Key words: CRE, FMDV, host susceptibility, RNA structure, virus variation

Introduction

The foot-and-mouth disease virus (FMDV), which causes severe vesicular disease in livestock, is a member of the Aphthovirus genus in the Picornaviridae family [2, 3]. The FMDV has high potential for antigenic and genetic variation; based on their induction of cross-protection in host animals, seven serotypes (A, O, C, Asia1, SAT 1, SAT 2, and SAT3) of FMDV have been identified [5, 20]. Additionally, advances in DNA sequencing have dramatically increased the rate at which genotypic and phenotypic variants of FMDV have been identified [4].

Replication and translation of FMDV RNA occur in association with the cell membrane in the cytoplasm of infected cells. The most critical step of FMDV replication is RNA-dependent RNA synthesis by 3D polymerase, which requires a regulatory network involving viral-encoded proteins (3B and 3D), various host factors, and a non-coding structural RNA element. The 5' UTR contains two highly structured RNA sequences; the cloverleaf, required for genome replication, and the internal ribosome entry site, which directs translation initiation[8]. RNA replication is carried out on membranous structures by viral RNA-dependent RNA polymerase in conjunction with other viral and cellular proteins and cis-acting replication element (CRE) [10, 19]. The viral RNA structure is critical for several essential functions, including replication, translation, and encapsidation [14]. Determining the structure of viral RNA has broadened our understanding of its involvement in the viral infection cycle [9, 24].

Replication of the FMDV is initiated by the 3B protein, which acts as a primer [7]. Uridylation by FMDV VPg occurs in a template-dependent manner and requires a small stem loop structure in the CRE as a natural template [17, 18, 22]. This reflects a common theme among many eukaryote-infecting viruses, which have evolved a variety of mechanisms to manipulate cellular transcription and trans-
lution machinery, in many cases via elegant RNA-centered strategies.

FMDV can spread through direct or indirect contact with infected animals and related products or by long-distance airborne transmission. Such spreading can occur in an extremely rapid manner for a variety of reasons, including the small amount of virus required to initiate infection, the large amount of virus excreted by affected animals, and the multiple routes of infection [23]. Additionally, the rate by which a population of viruses, including the FMDV, evolves can be influenced by genomic mutation rates, genomic architecture, and the speed of replication and recombination [1].

The biological significance of the CRE secondary structure in the 5’ UTR of FMDV is unclear. RNA functionality arises from its ability to fold into complex 3D structures and, often, its ability to change conformations to enable different functions, such as binding other types of RNA molecules or proteins. Interestingly, an extensive pan-flavivirus sequence analysis proposed that duplicated or repeated RNA motifs are associated with the acquisition of multiple hosts during viral evolution [9]. Experimental data from a dengue virus model indicated that RNA replication allows viruses to accumulate mutations that are beneficial in one host but deleterious in another, conferring robustness during host switching [24]. Additional studies have revealed the existence of different dengue virus RNA structures in two types of hosts [6, 15, 25]. FMDV infection is usually easier in cows than in pigs, but FMDV replication and transmission are more rapid in infected pigs than in infected cows. Since 2010, FMDV epidemics in Korea seem to be sensitive and specific to one type of host. In this study, we determined that the secondary structure of the FMDV CRE plays a role in determining the host specificity of an infection. These results, thus, assist in shedding light on FMDV evolution and host adaptation.

Materials and Methods

FMDV stock production

Viruses were isolated from LFBK (porcine kidney), ZZR-127 (goat fetal tongue epithelium) and BHK-21 (baby hamster kidney) cell lines obtained from the ATCC (LGC Standard). Virus isolation was performed according to the OIE manual (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.05_FMD.pdf). To produce an amplified stock of inoculum for viral serotypes A and O, two cell batches per serotype were inoculated with plaque forming units (PFUs) of field-collected vesicular fluid. For amplification of FMDV, cells were harvested after 24 hr infection and subsequently repeated freezing-thawing three times. The supernatants were collected in tubes and an equal volume of MEM with 25 mM HEPES was added to each tube before freezing at -70°C.

RT-PCR and FMDV RNA sequencing

FMDV RNA was extracted using an automatic RNA extraction machine (MagNA Pure 96, Roche), according to the manufacturer’s instructions. RNA was stored at -70°C until use. cDNA was synthesized using a PrimeScript™ II 1st strand cDNA Synthesis Kit (TAKARA). Briefly, a 10 μl reaction mixture was prepared containing 10 mM dNTP, 1 μl oligo dT primer (50 μM), 3 μl RNase Free dH2O, and 5 μl viral RNA. The mixture was incubated for 5 min at 65°C then cooled immediately on ice. Next, the reaction was mixed with 10 μl of a second reaction mixture containing 5X PrimeScript II buffer, 0.5 μl 40 U/μl RNase inhibitor, 1 μl enzyme, and RNase-free dH2O. The mixture was then incubated at 42°C for 45 min, then 70°C for 15 min.

The entire genome was amplified using AccuPower® ProFi Taq PCR PreMix (Bioneer, Korea), according to the manufacturer’s instructions, with nine overlapping pairs of FMDV-specific primers. RT-PCR products were analyzed by QiAxcel (Qiagen).

Purified PCR products were either sequenced directly or after cloning into the pGEM-T easy vector (Promega, USA). DNA sequencing was performed using an automatic DNA sequencer (ABI 3730) using the BigDye Terminator v3.1 cycle sequencing kit (ABI, USA). Analyses of sequence identity and divergence were carried out using BioEdit software (version 7.2.5.). PCR product sequences were assembled with SeqMan Pro software (DNASTAR, Inc., Madison, WI, USA) using default parameters.

Analysis of sequence arrangement

The FMDV genomic sequence was confirmed based on our sequencing results and the NCBI database. Viral gene sequences were arranged using a ClustalW multiple sequence alignment of full FMDV genome sequences. Amino acid sequence alignments of FMDV genes were also performed.
Table 1. Foot-and-Mouth Disease Viruses used in this study

<table>
<thead>
<tr>
<th>No.</th>
<th>Isolate</th>
<th>Serotype</th>
<th>Topotype &amp; Genotype</th>
<th>Host species</th>
<th>Collected year</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>O/AD/SKR/2010/C</td>
<td>O</td>
<td>SEA/Mya-98</td>
<td>Swine</td>
<td>2010</td>
<td>KF112887</td>
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<td>Swine</td>
<td>2010</td>
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<tr>
<td>3</td>
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<td>Asia/Sea-97</td>
<td>Swine</td>
<td>2010</td>
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</tr>
<tr>
<td>4</td>
<td>O/08/SKR/2011/C</td>
<td>O</td>
<td>SEA/Mya-98</td>
<td>Cattle</td>
<td>2011</td>
<td>KR401160.1</td>
</tr>
<tr>
<td>5</td>
<td>O/JC/SKR/2014/P</td>
<td>O</td>
<td>SEA/Mya-98</td>
<td>Swine</td>
<td>2014</td>
<td>KY089604</td>
</tr>
<tr>
<td>6</td>
<td>O/US/SKR/2014/P</td>
<td>O</td>
<td>SEA/Mya-98</td>
<td>Swine</td>
<td>2014</td>
<td>KY086465</td>
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<tr>
<td>7</td>
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<td>O</td>
<td>SEA/Mya-98</td>
<td>Swine</td>
<td>2016</td>
<td>Ky086466</td>
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<tr>
<td>8</td>
<td>O/GC/SKR/2016/P</td>
<td>O</td>
<td>SEA/Mya-98</td>
<td>Swine</td>
<td>2016</td>
<td>KY086466</td>
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<tr>
<td>9</td>
<td>O/BE/SKR/2017/C</td>
<td>O</td>
<td>ME-SA/Ind2001e</td>
<td>Cattle</td>
<td>2017</td>
<td>*APQA</td>
</tr>
<tr>
<td>10</td>
<td>O/JC/SKR/2017/C</td>
<td>O</td>
<td>ME-SA/Ind2001e</td>
<td>Cattle</td>
<td>2017</td>
<td>*APQA</td>
</tr>
<tr>
<td>11</td>
<td>A/YC/SKR/2017/C</td>
<td>A</td>
<td>Asia/Sea-97</td>
<td>Cattle</td>
<td>2017</td>
<td>*APQA</td>
</tr>
</tbody>
</table>

* Unpublished sequence by APQA
calculated that CRE sequences from pig isolates of FMDVs were extremely similar (Fig. 2D), while CREs from cow isolates of FMDVs showed large variations in divergence (Fig. 2C). Interestingly, comparisons of nucleotide sequences of FMDV isolates from cows, A/PC/SKR/2010/C, and two O serotypes from 2017 showed similar CRE variation patterns regardless of serotype. These results demonstrate that specific CRE variation patterns are associated with either different genotypes or host species.

Adaptive mutations of the full and CRE sequences of FMDV show grouped phylogenetic tree patterns.
A

Fig. 3. Maximum Likelihood tree showing phylogenetic relationships of FMDV isolates based on the complete genomic sequence (A) and CRE region sequence (B). Three trees were rooted using the FMDV type O strain (O/AD/SKR/2010/C). Bootstrap support values above 70% out of 1,000 replicates are shown near the major nodes. Horizontal branch lengths are drawn to scale.

Host species-associated differences in the RNA secondary structure of the FMDV CRE

Among FMDV epidemics in Korea over the last decade, those in 2014 and 2016 were primarily detected in pigs, while those in 2010, 2011, and 2017 were primarily detected in cows. For efficient FMDV replication in infected host cells, the RNA replication complex requires non-coding regulatory CREs, FMDV 3B/3D proteins, and host cell-specific replication factors. In addition, species-specific replication factors...
in cows or pigs may interact with the RNA replication complex of the FMDV while the CRE motif acts as a platform for the RNA replication complex. Therefore, we analyzed whether there are host species-dependent differences in the CRE secondary structure. The CRE secondary structures showed four different RNA structure patterns (Fig. 4). One CRE structural pattern was associated with pig-specific FMDV strains, including O/US/SKR/2014/Pig, O/JC/SKR/2014/Pig, O/GC/SKR/2016/Pig, and O/GJ/SKR/2016/Pig. The CRE associated with pig infections showed a 17 nucleotide-long loop, a 5 nucleotide-long stem, a heptagon, and two hexagons. CREs from cow infections showed three different RNA secondary structure patterns with different nucleotide lengths of loops and stems (Fig. 4). Additionally, heptagon structures were unique to the CREs of pig FMDV isolates and did not appear in cow isolates.

### Discussion

The functional cooperation of viral replication factors and host cell proteins plays a critical role in the viral replication of several picornaviruses. Based on these observations, FMDVs may acquire a distinct mechanism for efficient viral replication of their genomic RNA dependent on regulatory RNA elements in addition to host factors and FMDV non-structural proteins [15]. FMDV 3B protein shows weak association with the RNA replication complex of the FMDV genome, suggesting the assistance of another host cellular factor for the establishment of a strong viral replication complex. The formation of a complete functional complex is a rate-limiting step for FMDV replication [21]. For initial FMDV replication procedures, FMDV 3B and 3D proteins should recognize a cognate CRE RNA site [16]. These RNA secondary structures of FMDV CREs are crucial for acting as docking sites of host replication proteins as well as 3B and 3D viral proteins.

As the FMDV is an RNA virus with a single positive strand, it has shown high genetic variation in viral replication. For initial FMDV infection, only low levels of the virus may be required, and several rounds of viral replication with higher amounts of FMDV can transmit into a host animal with multiple virus variants. The rapid rate of FMDV replication induces an immune response in host animals within a short time, resulting in the decrease of other viral infections by related FMDV variants [12]. For the comparison of the RNA substitution of the FMDV, no equal distribution

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<tr>
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<td>6</td>
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<tr>
<td>stem (2) length</td>
<td>2</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>shape</td>
<td>heptagon</td>
<td>pentagon</td>
<td>pentagon</td>
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</table>

Fig. 4. RNA secondary structures of individual FMDV strains depend on the host species infected. The secondary structure of the CRE RNA of FMDVs was determined using the Fold Web Server with the lowest free energy calculation. The blue-colored box indicates relatively variable regions of RNA secondary structures of FMDV CRE.
was shown within the FMDV full genome. The highest gene variation was shown in the VPI region, resulting from the development of viral escape by vaccine usage and the host immune response. Even though there is only a small portion (less than 8%) of the VPI gene in the entire FMDV genome, it has been used in calculations of the phylogenetic relationship of FMDV variants, since the VPI site is important for host cell attachment and entry. In addition, VPI plays a critical role in the induction of the immune response and the determination of serotype specificity [5, 13]. The frequency of genetic variation of the non-coding region of the FMDV is lower than that of the coding region. CRE nucleotide substitutions show low rates of variation, but only one nucleotide variation within the CRE contributes to different RNA secondary structures for the recognition of CRE-binding proteins.

Here, we provide novel results regarding the epidemiological trends of FMDV outbreaks in Korea over a recent 10-year period, and provide viral determinants for host susceptibility. The endemic viruses from clinically infected animals showed different genetic variations dependent on the number of endemic years and the host species. Early identification of the host species of FMDV susceptibility can contribute toward efficient FMDV control programs, including vaccine application and quarantine countermeasures. The inter species transmission of FMDV between pig and cow might provide the genetic background of host specific susceptibility. However, in most of countries including Korea, since pig and cow farms were constituted of separated places, there were not research reports about FMDV inter-transmission of cow and pig. Generally, while cow might be highly infected by FMDV, pig could transmit FMDV stronger than cow.

The sequence-associated RNA secondary structure analysis of the FMDV CRE obtained from infected animal tissues may elucidate relationships between an outbreak and host susceptibility. Despite the presence of the same functional proteins in different host animals, the differential amino compositions of host cellular proteins may provide different CRE RNA recognition and discriminate in favor of specific host species for viral replication and transmission.

The pattern of genetic variation of the FMDV requires host adaptability for viral entry, intracellular replication, and establishment of the cytoplasmic viral assembly environment [11]. Accumulated information regarding the genetic variation of the FMDV may lead to the early determination of susceptible host species and provide molecular characteristics of emerging FMDV variants.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

References


초록: 구제역바이러스의 숙주 특이성 결정에 연관되어있는 구제역바이러스 cis-acting replication element 변이 분석 연구
강효린1, 성미소1, 구복경2, 정재훈1*
(1부산대학교 자연과학대학 분자생물학과, 2농림축산검역본부)

구제역바이러스(FMDV)는 피코나바이러스 과에 속하는 바이러스로서 야생과 가축화된 소와 돼지에 감염된다. FMDV는 제어되기 어려워 가축의 생산과 국제통상에 큰 장해가 되고 있다. FMDV RNA 게놈의 복제 과정에서 3D 중합효소가 특이적인 복제 기능을 담당하는데 게놈에 결합하는 부위가 매우 중요하다. 이 사실은 FMDV 게놈의 비코딩영역 내에서 3D 중합효소에 의해 인지되는 특이 RNA 구조가 관여함을 제시한다. 이 과정에서 cis-acting replication element (CRE)는 RNA 복제를 위한 개시에 필요하다. FMDV CRE는 15-17 뉴클레오티드의 고리와 이를 지지하는 이중가닥으로 형성된 점거-고리 모양을 가지며 이들을 구성하는 RNA 뉴클레오티드 서열의 차이가 다른 RNA 이차구조를 생성한다. CRE 이외에 FMDV 복제를 위해서 많은 바이러스와 세포 인자들이 단백질-단백질 결합과 단백질-RNA 결합을 통해 협조적인 네트워크를 만들어낸다. 이 연구에서 국내에서 2010년부터 2017년까지 구제역이 발생한 소와 돼지에서 FMDV를 분리하여 CRE 서열을 분석하였으며 이들 FMDV들은 A형과 O형의 유전자형을 가졌다.홍미들에게 국내 FMDV들의 CRE RNA 이차구조의 변이들은 바이러스 간의 계통유전학적 상관관계성을 알리며 특정 숙주 동물종의 FMDV 감염의 특이성을 밝혀주었다. 이로 토대로 국내 FMDV의 각 유전군의 분류는 독특한 기능적 CRE에 의해 특정시킬 수 있으며 새로운 유전적 계통의 진화학적 연속성을 특정하는 CRE 포타프의 구분과 연관성을 수 있다. 그러므로 CRE의 특이적 RNA 구조는 숙주 동물종 의존적인 FMDV 분류의 부가적인 단서로 활용할 수 있음을 제안한다. 이들 연구 결과들은 향후 FMDV 대량감염이 발생할 때 숙주동물종의 특이성을 CRE 서열로 초기에 정확히 분석하는데 도움이 될 것이다.