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# Foot and Mouth Disease Virus Genome

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## 1. Introduction

Foot-and-Mouth Disease Virus (FMDV) is a member of the Picornaviridae family of viruses, which includes viruses that cause a number of high consequence human and animal diseases in addition to Foot-and-Mouth Disease (FMD), such as hand-foot-and-mouth disease, herpangina, polio, and encephalomyocarditis. FMDV infects domestic and wild cloven-hoofed animals, including bovine, caprine, ovine and swine species that are vital to the livestock industry. Depending on host and virus characteristics, FMD exhibits a broad range of clinical presentations resulting in significant morbidity. Generally, FMD produces fever and soreness, excessive salivation, loss of appetite and large vesicles on the feet, nose and tongue 24 to 72 hours post-infection (hpi). In some cases, complete prostration accompanied by the loss of hooves occurs (for review see refs. 1-4). Although mortality rates are typically low and usually associated with young or immunocompromised animals, the economic consequences of an FMD outbreak, such as dramatic decreases in livestock productivity and banning of the export of animals and animal products, are so significant that FMD is one of the most threatening diseases of domestic animals in the world. In many developing regions of Asia, Africa and South America, FMD is enzootic. Global economic activities and transboundary movement of people and animals presents a significant risk of accidental introduction of FMDV into previously FMDV-free countries. Additionally, terrorist groups may intentionally introduce FMDV into a country that is FMD-free and does not vaccinate against the virus (refs.5-8 and <http://www.oie.int>; <http://iah.bbsrc.ac.uk/virus/Picornaviridae/Aphtovirus/fmd.htm>).

## 2. Foot-and-mouth disease virus

Like all picornaviruses, FMDV is a non-enveloped virus with icosahedral symmetry and contains a single-stranded, positive-sense RNA molecule of approximately 8500 nucleotides (nt). The viral particle is small in size (approximately 30 nm diameter) and is composed of 60 copies each of proteins 1A, 1B, 1C and 1D (also known as VP4, VP2, VP3 and VP1, respectively) assembled in groups of increasing complexity. A single cluster of structural proteins, known as the *protomer*, involves one copy each of 1A, 1B, 1C and 1D. Five protomers assemble together to form a *pentamer*, which are then assembled into groups of twelve to form the complete viral capsid (9, 10).

FMDV RNA possesses genetic characteristics, such as positive polarity and a polyadenylated 3' end, that allows it to act as messenger RNA (mRNA) *in vitro* and *in vivo*, and therefore should be considered a potentially infectious agent. RNA extracted from field

samples has been used to produce infectious viruses using *in vitro* cell electroporation techniques. The rescued viruses were highly pathogenic in natural hosts and could be characterized using ELISA and full genome sequencing. This feature of the FMDV genome allows for manipulation of cDNA copies to study the genetics of pathogenesis and observe the phenotypic effects of mutations and other genomic alterations. This application of “reverse genetics” to construct chimeric and recombinant FMDVs has led to the discovery of several determinants of viral replication, host recognition and virulence (3, 4, 11). Continued use of reverse genetics will enhance current models of FMDV pathogenesis and further progress towards therapies and vaccines.

### 3. FMDV genomics publications

Analyses have been performed on the complete P1 polyprotein, the genomic region encoding all four structural proteins that compose the viral capsid (1A, 1B, 1C and 1D). However, most of the work published regarding FMDV genomics is limited to the coding region of capsid protein 1D (also known as VP1). This information has been used to analyze variability, selective pressures and immunogenicity of FMDV. Phylogenetic analysis employing 1D sequences and a 15% nucleotide difference as a cut-off organizes FMDV strains into major groups or genotypes. Interestingly, the genotypic information obtained with approximately 636 nucleotides of VP1 completely matches the phylogenetic results obtained when 2208 nucleotides of the complete P1 polyprotein are used (12, 13). Genetic lineages subsequently are geographically bound and are described as “topotypes”. The viral capsid, notably the 1D protein, harbors immunogenic epitopes that are critical for neutralization of the virus. Hence, the topotype classification system has extraordinary value for vaccine selection.

The development of high-throughput sequencing techniques has allowed for complete genome sequencing of FMDV, significantly improving our understanding of infection, host range, and transmission. The use of complete FMDV genome sequences in phylogenetic studies has revealed much more complex epidemiological relationships between isolates than previously thought. Full genome comparisons suggest that the epidemiology of FMDV is heavily impacted by recombination; it also has led to the discovery of novel genetic lineages containing genomic sequences that appear equally distant from SAT and Euroasiatic lineages of the virus. Furthermore, complete genome sequencing has enhanced the discovery of FMDV variability, sequence conservation and universal genetic motifs that affect its virulence and transmission. Complete genome sequence analysis of FMDV isolates collected during the August 2007 outbreaks in England identified the initial and intermediate sources of the outbreaks, demonstrating the value of complete sequence analysis when examining virus phylogeny, an accomplishment previously impossible using partial genomic sequences (14, 15, 16).

## 4. Genome structure and proteins

### 4.1 FMDV genome

Upon entrance into the host cell the virus particle dissociates and the RNA is released into the cytoplasm. The genome of picornaviruses functions as a messenger (+) RNA, polyadenylated at its 3'-end and covalently linked to a small protein (VPg) at its 5'-end.

Translation occurs as a single polypeptide precursor (ORF) that is cleaved into functional proteins, mostly by virally encoded proteases (3, 10, 17).

Primary processing of the FMDV ORF results in three large intermediate polyproteins (L/P1, P2 and P3). Protease cleavage by FMDV proteins L, 3C and 2A produces smaller sub-products and 12 final mature proteins: L, 1A, 1B, 1C, 1D, 2A, 2B, 2C, 3A, 3B, 3C and 3D (Figs. 1 and 2). As mentioned above, 1A, 1B, 1C and 1D are the structural proteins that form the viral capsid. FMDV 2B and 2C proteins localize to ER-derived vesicles, the site of viral replication, but their functions remain poorly understood. 3A is thought to be a multifunctional integral membrane protein that enhances viral RNA synthesis and exhibits host-related markers. Unique to FMDV are three non-identical copies of the genome-linked 3B, a protein required for viral RNA replication. Finally, 3D encodes the viral RNA-dependent RNA polymerase, and along with 3A, co-localizes with ER membrane-associated replication complexes.

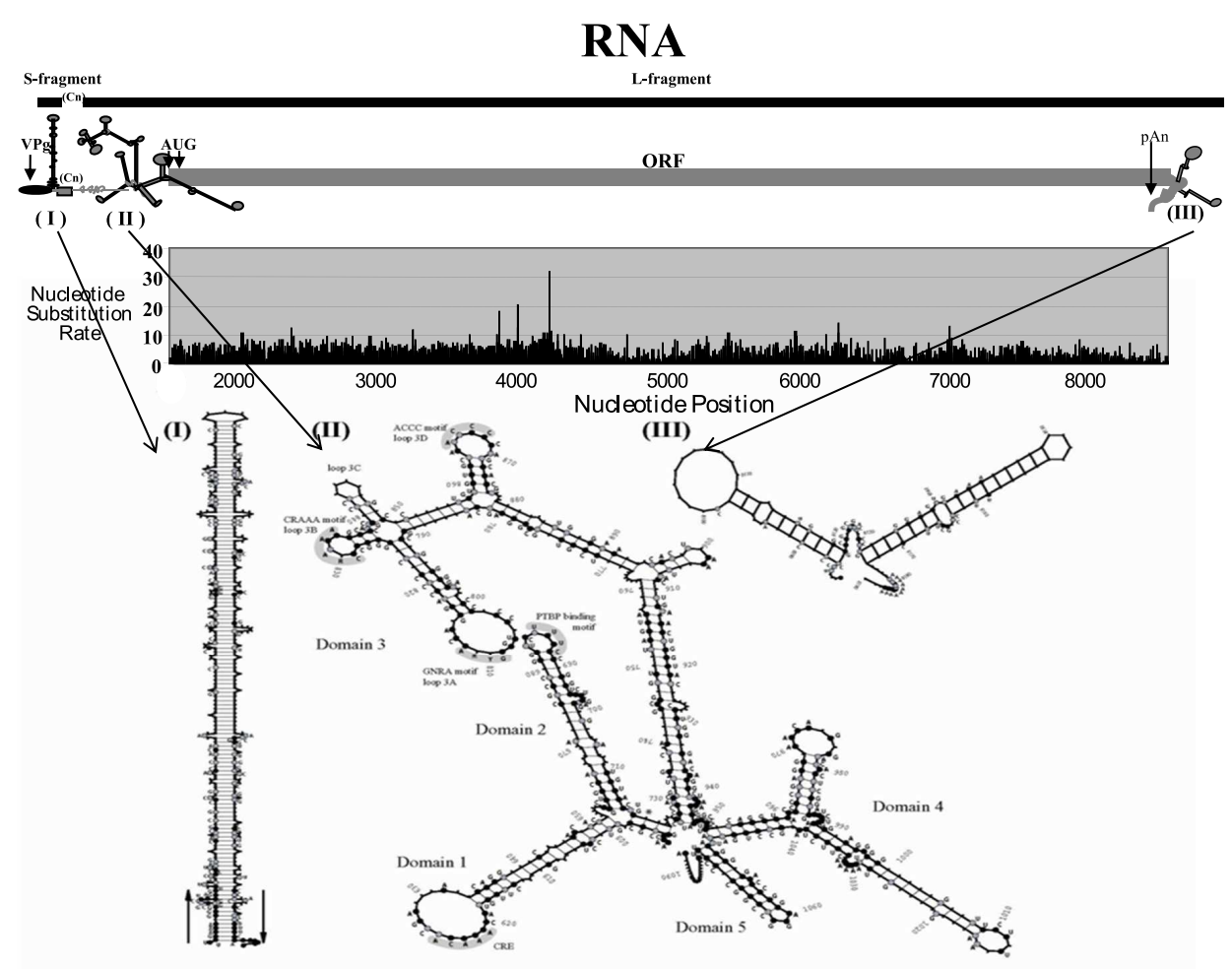


Fig. 1. FMDV RNA genome representation with detailed description of the UTR elements and its predicted secondary structure: (I) S-fragment; (II) IRES; (III) 3' NCR. Also includes a graphic representation of the distribution of variability within the complete coding region (ORF), expressed as rate of substitutions per nucleotide site of a Clustal W multiple alignment of all serotype FMDV full length genomes (ref 14 for more detailed view).

The nomenclature used for FMDV proteins is similar amongst all picornaviruses and is based on their position in the viral RNA genome (18). However, it does not imply a conserved function of the proteins across all genera. In fact, there is increasing evidence that despite sharing similar genome organization and protein names there can be significant differences in functionality. For instance, FMDV encodes a 3A protein that is 50% longer than the equivalent protein encoded by poliovirus; FMDV also harbors three copies of 3B in contrast to a single copy in poliovirus. Additionally, the role of the poliovirus 3A protein in immune evasion and persistent infection seems to be played by FMDV 2BC.

Traditionally, the FMDV genome is classified into coding (ORF) and non-coding regions (NCRs) that distribute along three defined genomic intervals: (i) the 5' untranslated region (5'-UTR), which contains non-coding nucleic acids that carry many regulatory elements; (ii) the protein coding region (ORF), which includes both structural and non-structural proteins; and (iii) the 3' UTR or non-coding region, which also carries regulatory functions and a poly(A tail).

## 4.2 FMDV 5' UTR

The 5'-UTR of FMDV is unusually long and highly variable, both in length and nucleotide composition. It includes a number of structural and functional elements that are critical for the replication and biology of the virus (17, 19, 20). The role(s) of many FMDV RNA domains in the 5' UTR are poorly understood, but several have been analyzed and are described herein. Ordered from the 5'-terminus of the molecule (Fig. 1), the following regions have been defined:

A highly structured small fragment or "S-fragment" of about 370 residues of unclear function. Evidence suggests that the S-fragment has a critical role during RNA replication (Fig. 1-I).

An internal polyribocytidylic, or poly(C), tract of 100 to 400 nucleotides, comprised predominantly of cytosine residues. Unusual among picornaviruses, it has been described as an element related to virulence, but subsequent studies using infectious clones (ICs) suggest the contrary. Its biological function is not well understood (21).

A pseudoknot region, also of unknown function and variable length. This region contains significant deletions in some FMDV isolates, and its presence has never been linked to any specific biological function.

A cis-Replicating Element (cre) or 3B-uridylylation site (bus), conforms of a stem-loop structure containing a conserved AAACA motif, functions as a template for addition of U residues to the protein primer 3B. It is critical during transcription in order for circularization of the viral RNA to occur (22).

A type-2 IRES of about 440 nt, comprised of five major domains, H-L (Fig 1-II). The IRES facilitates the internal initiation of protein synthesis in a CAP-independent fashion, allowing it to mediate ribosome recruitment to an internal site within the viral RNA (23). This process is facilitated by eukaryotic translation initiation factors (eIFs). Initiation of translation by the IRES begins with specific binding of the central domain initiation factor, eIF4G, to the J-K domains, which is stimulated by eIF4A. Then, these initiation factors induce a restructuring of the region and promote recruitment of ribosomal pre-initiation

complexes. PTB and ITAF45 trans-acting factors are also required to stabilize the active conformation. Both binding of eIF4G to the IRES and IRES-directed translation are significantly impaired by mutations that impact the integrity of the double-stranded secondary structure. In fact, the primary sequence within the IRES of different FMDV isolates can be up to 50% variable and still retain similar overall secondary structure using compensatory base changes in helical elements.

4.3 FMDV coding region (ORF)

Protein synthesis starts at one of two functional in-frame AUG codons, separated from each other by an indispensable but highly variable tract of approximately 80 to 84 nucleotides. The long ORF that follows the AUG codon encodes a polyprotein of about 2330 amino acids (aa), although length and composition among natural isolates and even among passaged viruses can be variable. Although the polyprotein intermediaries of processing are biologically important, the current discussion will concentrate only on the twelve final protein products. For a complete review of cleavage sites, biologically critical residues, and variability/conservation between serotypes, see ref. 3, 4, 18.

Components of the polyprotein, from 5' to 3' (Fig. 2):

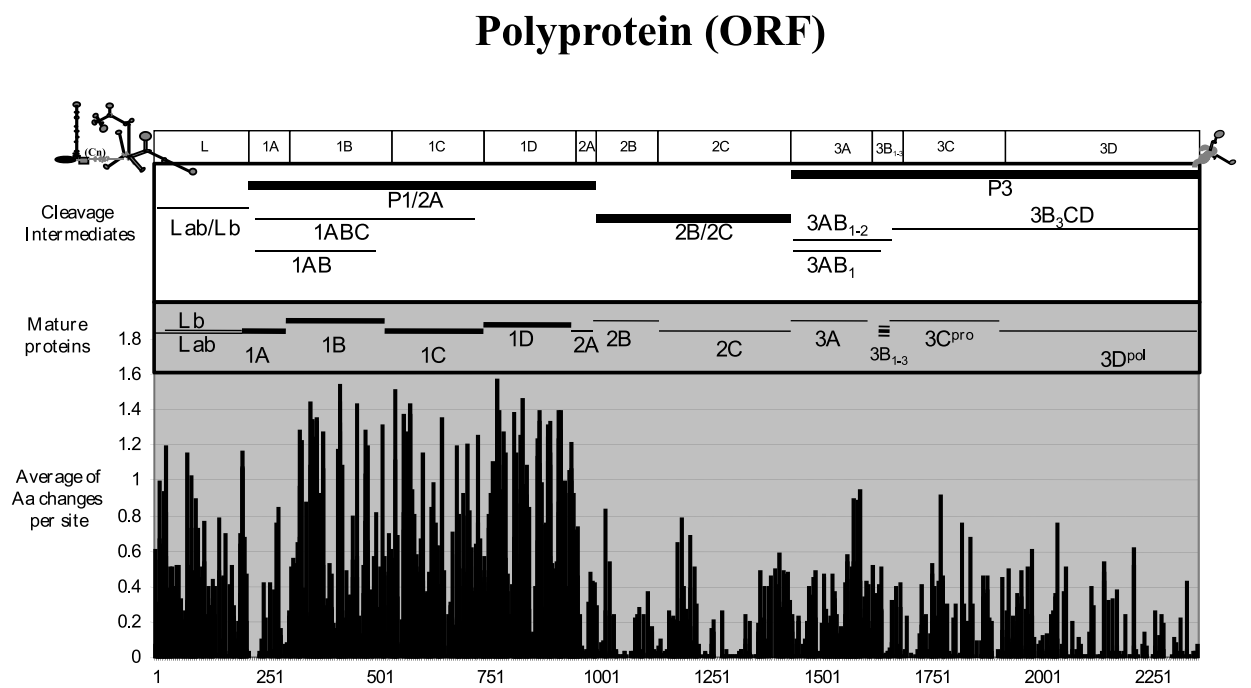


Fig. 2. Upper panel: schematic representation of FMDV genome and poly-protein coding region including protein-encoding regions, cleavage intermediates and mature protein products. Lower panel: schematic representation of the non-synonymous (amino acid) substitutions per site within the whole poly-protein (ORF).

4.3.1 Leader protease (Lpro)

Two in-frame AUG codons allow for two different initiation events within Lpro, producing two forms of the protein, named Lab and Lb. Both proteins catalyze their own cleavage at



their C-terminus from the rest of the polyprotein as well as cleavage of the initiation factor eIF4G (p220) of the CAP-binding complex eIF4F, contributing to the shut-off of host cell protein synthesis. Approximately one-third of cell ribosomes initiate at the first AUG (AUG1, of Lab) site, while the majority of ribosomes start translation at the second AUG codon (AUG2, of Lb). It is unknown why ribosomal preference for the second AUG exists. Both proteins also limit the host innate immune response via inhibition of interferon beta (IFN- $\beta$ ) mRNA expression. *L(pro)* localizes to the nucleus of infected cells and disrupts the integrity of the nuclear factor NF- $\kappa$ B using mechanisms that antagonize the cellular innate immune and inflammatory responses to FMDV infection.

#### 4.3.2 P1 coding region

The P1 polypeptide sequence begins immediately downstream of the *Lpro* protein. Included within P1 are the four capsid proteins: *1A*, *1B*, *1C*, and *1D* (also known as *VP4*, *VP2*, *VP3*, and *VP1*, respectively). With the exception of *1A*, which is excluded from the virion surface, capsid proteins (*1B*, *1C* and *1D*) are involved in antigenicity and binding to a subset of RGD-dependent integrins and heparin sulfate proteoglycan receptors on the cell surface (reviewed in Ref. 24).

FMDV structural proteins are involved in capsid assembly and stability, virus binding and antigenicity. Despite these essential characteristics, there is a high degree of flexibility in the primary sequence of most of these proteins. The structural proteins exhibit the highest rates of nucleotide and amino acid (aa) variability among all viral proteins, likely a response to intense selective pressures. *VP4* is an exception to this observation, since 73% to 84% of its nucleotide sequence is conserved among all FMDV isolates.

The only internal capsid protein, *1A*, carries a swine-specific immunodominant and heterotypic T-cell antigenic site that is capable of providing help to a B-cell epitope when in tandem. Within the amino half of *1A*, a conserved myristate binding site exists.

Structural protein *1B*, or *VP2*, plays a critical role in capsid stability and particle maturation, supported by the observation that 47% of its amino acids are invariant between and among different FMDV serotypes. At least 3 T-cell epitopes have been identified within *1B*, exemplifying its immunogenicity. A number of important conformational neutralizing epitopes and one T-cell epitope have also been identified in protein *1C* (*VP3*).

The best known FMDV protein is *1D*, also known as *VP1*. *VP1* is the most variable region of the FMDV genome; only 26% of its aa are universally conserved between serotypes. Many of the residues known or suspected to be critical for cleavage or other functions are located within invariant sequence motifs, indicating that the critical function of those residues may be contextual and require other specific residues. Protein *1D* is responsible for virus attachment and entry, protective immunity and serotype specificity. A major, non-essential immunodominant site is located within the so-called G-H loop of *VP1*. This loop appears highly disordered in X-ray diffraction patterns of crystallized virions, but it is known to protrude from the capsid surface when the capsid is bound to an antigen-binding fragment (Fab). After binding of a cellular integrin receptor to the RGD motif in *1D*, FMDV utilizes endocytosis to infect the cell. Viral replication commences when the viral capsid dissolves, allowing the release of RNA into the cell cytoplasm. Viruses that have sequences similar to the RGD motif can infect cells via different integrin receptors and can induce disease and

transmission to susceptible animals. Other critical aa residues have been identified, such as the methionine (Met) at position 54, whose change to isoleucine (Ile) affects processing of precursor P1, decreasing production of VP1 and accumulation of VP1 precursor proteins. Although not in direct contact with the VP1-VP3 cleavage site, residue M54 of VP1 is exposed at the virion surface and it is close to an antigenic site within the B-C loop. Residues within the 1AB cleavage pocket and a 1C histidine (His-142- $\alpha$ -helix charge-dipole interaction at the twofold axes of symmetry between *pentamers*) play a role in acid-induced disassembly of the capsid.

#### 4.3.3 P2 coding region

Most of the non-structural proteins (NSPs) are found within the P2 and P3 coding regions. These polypeptides and mature proteins are involved in RNA replication and viral maturation, although their specific roles remain to be elucidated. FMDV 2A is an 18 aa peptide that induces P1/2A polypeptide release from the rest of the genome through modification of the cellular translation apparatus. Generating the C-terminus of 2A and the N-terminus of 2B does not involve a protease, but rather cleavage of the ester linkage of peptidyl-tRNA within the peptidyl-transferase center of the ribosome during translation, a phenomenon termed 'StopGo'. The functional motif of 2A resides in a highly conserved aa sequence in its carboxy-terminal portion. This co-translational dissociation of the polyprotein and immediate recovery has been widely applied to develop research tools and gene therapies. The 2A protein is released from P1 by cleavage with the 3C viral protease in a later stage of processing. However, its function as an independent protein is not yet understood.

Little is known about the function of the FMDV 2B protein. It is a small hydrophobic protein that, upon individual expression, is localized to the endoplasmic reticulum (ER) and the Golgi complex. Differing from other picornaviral 2B proteins, FMDV 2B has minimal effects on Ca(2+) homeostasis and intracellular protein trafficking. However, it does cause accumulation of ER proteins in large vesicular structures around the nucleus. A trans-membrane domain has been predicted between aa positions 120 and 140, supporting its involvement in vesicles and membrane-related stages of viral infection. Its expression in cells enhances membrane permeability and has been implicated in cytopathic effect. Only 37 of the 154 aa that compose the 2B protein are variable, and even those are restricted to one or two possible aa's, illustrating the great constriction of 2B protein variability.

Protein 2C is a highly conserved peptide with ATPase and RNA binding activity that is 318 aa in length. It has been assigned to the SF3 helicase family of AAA+ ATPases. In infected cells 2C is involved in the formation of membrane vesicles where it co-localizes with viral RNA replication complexes. Its 18 nt ATP-GTP binding motif is highly conserved in all serotypes. This sequence motif is generally referred to as the "A" consensus sequence or the P-loop, and is found in many protein families, such as thymidine kinases, ATP-binding proteins involved in active transport, DNA and RNA helicases, etc.. Protein 2C is involved in RNA synthesis and is the site of mutations that confer resistance to guanidine hydrochloride. Substitution of arginine 55 to tryptophan (R55W) within 2C mediates an increase in the extracellular release of viral RNA without a detectable increase of total viral RNA.



#### 4.3.4 P3 coding region

FMDV protein 3A is a membrane-associated protein that localizes to a reticular structure. Some studies suggest that 3A influences host-range; for example, the amino acid substitution glutamine 44 to arginine (Q44R) in 3A, either alone or in combination with the replacement of isoleucine 248 with threonine (I248T) in 2C, was sufficient to give FMDV the ability to produce lesions in guinea pigs. Also, a 10 aa deletion and a series of substitutions (accumulated over the following 29 years) that surround the deletion were described to be a primary determinant of restricted growth of O/Taiwan 97 on bovine cells *in vitro* and as a contributor to bovine attenuation of O/Taiwan 97 *in vivo*. Subsequent experiments demonstrated that this deletion on its own does not contribute to porcine tropism of the virus, but that genome-wide changes (in addition to the deletion) produce the porcophilic phenotype of current Asian viruses within this lineage.

The 3B region codes for 3 non-identical copies of the VPg protein. Covalent linkage of VPg occurs via a tyrosine (Y) residue to the 5' end of the positive-sense RNA viral genome and to the nascent viral plus- and minus-stranded RNAs. This protein serves as a primer for the initiation of RNA replication and plays a role as an encapsidation signal. This priming step requires uridylation of the VPg peptide by the viral polymerase 3D and other viral or host cofactors. It remains to be elucidated why FMDV is the only picornavirus that encodes 3 tandem repeats of the VPg protein within the 3B coding region. Limited studies suggest it may be a critical component of the viral replication complex, enhancing transcription efficiency of the viral genome. Engineered FMDV infectious clones with either severe domain disruption or deletion of individual 3B proteins do not exhibit decreased infectivity *in vitro*, nor do they alter clinical disease in cattle or swine. Only clones encoding a single copy of VPg seem impaired in replicative competence. Interestingly, these mutant FMDVs did not produce plaques in BHK-21 tissue culture but produced a mild disease in swine and cattle.

The viral proteinase, 3Cpro, is a serine protease that catalyzes ten of the thirteen cleavages necessary to complete FMDV polyprotein processing. Its protease activity also affects host cell transcription since 3Cpro is responsible for the cleavage of the cellular histone H3 as well as the elongation factors eIF4G and eIF4A, resulting in cessation of host cell transcription. Crystal structure analyses indicate that FMDV 3Cpro adopts a chymotrypsin-like fold in the characteristic fingers, palm and thumb subdomains, with the presence of an NH2-terminal segment encircling the active site. The necessity of this tridimensional structure imposes serious restrictions on amino acid variability.

Replication of the RNA genome of the virus, via negative strand intermediates, involves an RNA-dependent RNA polymerase, 3Dpol. Several specific aa's have been determined as essential for maintaining the functional integrity of the polymerase. A NTP-binding motif and hydrophobic antigenic regions have also been described within 3Dpol.

#### 4.4 FMDV 3' UTR

The 3' end of foot-and-mouth disease virus is required for viral infectivity and stimulates IRES activity. It is composed of two distinct elements: a 90 nt untranslated region (3'-NCR), and a poly(A) tract. The 3'-NCR has a highly conserved structure composed of two stem-

loops (SL-1 and SL-2) that interact with viral and host proteins during RNA replication. The poly(A) tract is generally heterogeneous in length and has an important structural role during replication. Independent deletions within the two predicted stem-loop structures of the 3'-NCR have provided information about potential functions. Deletion of SL2 was lethal for viral infectivity *in vitro*, while removal of SL1 negatively impacted viral growth kinetics and impaired negative-strand RNA synthesis, down-regulating genome replication. Studies examining the *in vivo* phenotype of these mutant viruses in pigs suggest that deletion of SL1 may contribute to FMDV attenuation, supporting the potential of RNA technology for the design of new FMDV vaccines. The 3' end of FMDV RNA establishes two distinct strand-specific, long-range RNA-RNA interactions: one with the S region and another with the IRES element. The S region interacts with each of the stem-loops, and such interaction is dependent of the poly(A) conformation.

## 5. FMDV cell entry and genome replication

FMDV initiates infection by binding to integrin receptors via an Arg-Gly-Asp (RGD) sequence found in the G-H loop of the structural protein VP1. The particle dissociates into pentamers at mildly acidic pH and the RNA is liberated into the cytoplasm of the infected cell. FMDV uses standard picornavirus cell entry mechanisms, forming 'altered' particle intermediates thought to induce membrane pores through which the genome can be transferred across the endosome membrane. Induction of viral RNA translation and cessation of cellular RNA translation occurs concurrently. The synthesis of cellular proteins is prevented by viral proteases that cleave cellular elongation factors, inhibiting CAP-dependent translation. The viral proteins required for replication are immediately obtained from translation of the positive-sense viral RNA. These proteins also synthesize negative-sense transcripts based on the positive-sense RNA template. The negative-sense RNA then becomes the template used to synthesize *di novo* positive-sense viral genomes (24). Anti-sense RNA is found at a one hundred-fold less concentration than sense strands in infected cells, suggesting that each negative-sense strand may serve as template for the synthesis of many positive-sense strands. Genome copying occurs via a complementary negative-sense RNA template and the formation of two replicating positive-sense strands. Partially double-stranded replicative intermediates may also be involved (review in ref. 22-24).

FMDV RNA replication is initiated by the covalent attachment of an uracil monophosphate (UMP) molecule to the hydroxyl group of a tyrosine within the terminal VPg protein. This reaction is catalyzed by the virally encoded RNA-dependent RNA polymerase, 3D. The enzyme performs this operation, together with other viral and probably host proteins, in the cytoplasm of the host cell. Cytoplasmic RNA Helicase A (RHA) plays an essential role during replication of FMDV, interacting with the S fragment and the viral 2C and 3A proteins, as well as with cellular PABP, promoting the assembly of ribo-nucleoprotein replication complexes at the 5' end of the genome. Eukaryotic initiation factors (eIFs) are required for internal translation initiation at the internal ribosome entry site (IRES), an action common to all picornaviruses. The eIF4B is an RNA-binding protein that stimulates the ATPase and helicase activities of eIF4A and strengthens the mRNA-rRNA-tRNA interactions at the initiation codon. The eIF4A is an ATP-dependent RNA helicase; it is believed to unwind RNA secondary structure and is the prototypic member of the DEAD box family of helicases. The eIF4B is present both as part of the eIF4F complex bound to

eIF4G, and also free in the cytoplasm. The helicase activity of eIF4A is strongly stimulated by eIF4B. The cleavage of eIF4G releases the N-terminal domain that contains the binding sites for eIF4E and the poly(A) binding protein. The residual portion of eIF4G, which is sufficient for IRES-directed translation, retains two binding sites for eIF4A and binding sites for eIF3 and Mnk-1. Thus eIF4G is a bridge between the mRNA and small ribosomal subunit. The eIF4B is incorporated into ribosomal 48S initiation complexes via the FMDV IRES. In contrast to the weak interaction of eIF4B with capped cellular mRNAs and its release upon entry of the ribosomal 60S subunit, eIF4B remains tightly associated with the FMDV IRES during formation of complete 80S ribosomes. Binding of eIF4B to the IRES is energy dependent, and binding of the small ribosomal subunit to the IRES requires the previous energy-dependent association of initiation factors with the IRES. The interaction of eIF4B with the IRES in 48S and 80S complexes is independent of the location of the initiator AUG and thus independent of the mechanism by which the small ribosomal subunit is placed at the actual start codon, either by direct internal ribosomal entry or by scanning.

Final assembly of the viral capsid and encapsidation of the viral RNA occur by mechanisms that are still obscure. Two hypotheses describe potential mechanisms of pentamer assembly into pro-virions. One idea postulates that the RNA is inserted into the virion after assembly of the capsid, while the other theory proposes that the viral RNA interacts directly with the pentamers to form the pro-virion prior to capsid formation.

## 6. Genetic variability of FMDV RNA

Due to the absence of proofreading-repair activity by the viral replicase, FMDV RNA genome replication is highly error-prone. The high mutation rates result in populations that consist of genetically related but non-identical viruses known as *quasispecies*. The *quasispecies* concept maintains that a viral population consists of a 'swarm' of genetic and phenotypic variants in perpetual renewal as genome replication proceeds in an infected host. The consensus nucleotide sequence of FMDV isolated from a clinical sample derives from a multimodal population of similar but distinct viruses; often the exact consensus sequence obtained does not exist within the population, but is a reflection of many co-existing *quasispecies*. The existence of quasispecies populations may explain the dramatic genetic plasticity observed in disease-causing RNA viruses, supporting pathogenic adaptations that expand their host repertoire and virulence profile (Review in Ref. 26-29).

Several *in vivo* experiments report the generation of highly variable FMD viruses from single animals during infection studies. These observations may have been influenced by molecular host factors and/or selective pressures indirectly incurred from lab methodologies (see ref. 28-32). However, additional controlled experimental infections in pigs confirmed these observations in every passage of *in vivo* infection. Interestingly, the location and nature of the genetic variation was not the same as *in vitro*-acquired differences (see ref. 31, 32, 33, 34, 37), including the estimated number of substitutions per nucleotide. Recently, a study conducted during the United Kingdom 2001 epidemic demonstrated that nucleotide changes occur throughout the genome at a rate of  $2.26 \times 10^{-5}$  nucleotide substitutions per site per day (95% confidence interval [CI],  $1.75 \times 10^{-5}$  to  $2.80 \times 10^{-5}$ ) and nucleotide substitutions accumulate in the consensus nucleotide sequence at an average rate of 1.5 substitutions per farm infection. Data obtained from outbreaks like the 2001 epidemic

support the experimental observations, demonstrating the role of host-related selective pressures on the variability and evolution of FMDV (35).

Comparative genomics studies using full-length sequences representative of all seven serotypes have identified highly conserved genomic regions, indicating functional constraints for variability as well as as-yet undefined motifs with likely biological significance (14, 34). At least 64% of all nt sites within the FMDV genome are susceptible to substitution, including compensatory substitutions. It is important to clarify that most of the “variant” or substitutable residues within the FMDV genome mutate in response to detrimental effects produced by mutations elsewhere in the genome. But most importantly, it indicates that at least 46% of the nucleotides are indispensable for FMDV survival; replacement of any of the known critical residues renders non-viable progeny.

In support of the comparative genomics analyses, sequence studies demonstrate that the most distantly related pair of FMDV isolates to-date do not differ more than 22% from each other. Within one serotype the differences are less than 15%. Although sequences have been intensely analyzed in terms of similarity and divergence, the genetic bases of most biological traits of FMDV remain to be discovered. Such analysis would benefit from initial studies examining the conserved regions within the ORF; notably, regions 2B and 3C exhibit the highest percentage of invariant nt (61% and 59%, respectively) and amino acids (76%) in the genome.

The most variable parts of the translated FMDV genome, like *Lpro*, 3A, 3B and the structural proteins (1B, 1C, and 1D) suggest that these proteins are subjected to strong selective pressures. Additional studies and characterization will reveal important molecular markers and signatures of epidemiological and forensic value. This information can be used to facilitate development of novel vaccines containing molecular markers that allow for differentiation of vaccinated and infected animals.

## 7. FMDV RNA recombination and evolution

Genomic comparisons of full-length sequences have been very useful to the understanding of FMDV evolution. Computer programs have been developed and used to estimate various parameters of evolution. The CODEML program is one of most popular, widely used to obtain the  $\omega$  parameter ( $\omega = dN/dS$ ), an indicator of selective pressure that considers and compares several models of evolution. For FMDV, CODEML  $\omega$  rates obtained from different substitution models indicated that a few clusters of codons in *Lpro*, 1D, 3A and 3B may undergo diversifying selection. Evidence of positive selection has been identified in complete capsid sequences from all serotypes. Results suggest that novel antigenic variants benefit from a selective advantage in their interaction with the immune system, possibly throughout the course of an infection and/or during transmission to individuals with previous exposure to antigen (see ref. 14, 36-38).

Analysis of amino acid usage at sites under positive selection indicates that this selective advantage can be conferred by amino acid substitutions that share physical and chemical properties. Besides genetic drift, there is increasing evidence that recombination is an important mechanism of FMDV evolution. Using different recombination detection methods among the publicly available FMDV complete genome sequences, the large number of recombinant isolates suggests that horizontal recombination of sequences is common and



probably advantageous in terms of fitness costs. Interestingly, the distribution of recombination breakpoints was found to be largely nonrandom (37, 38). Results suggest that genome regions encoding the structural proteins are functionally interchangeable modules, as can be deduced from evidence that the structural and nonstructural coding regions of picornaviruses evolve largely independently of one another (see ref. 14, 37-40).

Recombinant viruses may derive from an animal that is co-infected with different virus variants while also harboring viruses from a previous persistent or sub-clinical infection. We are still not very knowledgeable about subclinical and persistent infection. Indications of differential susceptibility for developing a subclinical course of disease has been observed in many instances: buffaloes and cattle present with different disease manifestations, with breed affecting severity (see ref. 41); sub-clinical symptoms in sheep variable and goats make detection of FMD difficult in those species (see ref. 42, 43, 44); and establishment of persistent infection in ruminants has been demonstrated (see ref. 45). Most interestingly, pigs experimentally infected with a highly virulent porcine-tropic strain (OTw97) exhibited gradual loss of virulence and the establishment of a subclinical infection upon serial passage in the absence of FMDV-specific antibodies (38). However, experimental evidence of possible mechanisms of transmission and its effects on the FMDV genome are sparse; additional controlled experimentation in this field is required (32, 33, 38).

The initial size of a virus population strongly influences evolution and replication fitness. While *in vitro* large population passages often result in fitness gains, repeated plaque-to-plaque transfers result in average fitness losses, known as Muller's Ratchet effect. On the contrary, experimental infection of a natural host with FMDV seems to require a relatively low number of FMDV particles to produce clinical disease. *In vivo* studies suggest that horizontal transmission of FMDV may be achieved with a low number of infectious particles. In this scenario, recombination events may rescue defective genomes (Muller's Ratchet) yielding a significant number of virulent viruses that spread into new hosts. In this manner, FMDV perpetuates within and between natural hosts and reservoirs, recovering the replicative capability of previously defective particles.

Additionally, recombination within the non-structural genome regions, potentially modifying the virulence of the virus, may be involved in the success of the new sub-lineage to regain infectiousness. It can also explain why phylogenetic analyses restricted to VP1 sequences appear to represent evolutionary cul-de-sacs and why they often reveal re-emergence of previously extinguished VP1 genetic lineages.

Recombination might be a decisive factor in the production of escape variants. Strong immunity includes multiple B-cell and T-cell epitopes that produce efficient humoral and cellular immune responses. Such an ample response is obtained after recovery of natural infection but is difficult to obtain as the immune response evoked by vaccination. Both B and T-cell epitopes have been identified in structural and non-structural proteins of FMDV. Some are highly conserved, but others are highly variable. Therefore, it is possible that re-arrangement of the antigenic display is one of the mechanisms to escape host immune response for FMDV, and that recombination is one major player in such re-arrangement of antigenic markers.



## 8. Final remarks

- i. A new vaccine generation marked and targeting systemic and mucosal immunity is urgently needed for FMDV global eradication plans. Control of FMD is based on two major strategies: the slaughtering of affected and contact animals (the so called 'stamping out' procedure) or the regular vaccination of the major host species for FMDV. Unfortunately, classical vaccines cannot prevent the establishment of persistent FMDV infection in cattle. As an alternative to the conventional inactivated vaccines, the use of attenuated antigenically marked virus able to induce a solid and durable immunity through replication in the animal is highly desirable. FMDV escapes from vaccine production plants or diagnostic and research facilities, like what happen in UK 2007 ( O1BFS 1860/UK/67) highlight the need for an alternative to the handling of large amounts of virus because of the danger of virus escaping from vaccine factories. Also, classical, inactivated whole-virus vaccines may be at the origin of outbreaks if inactivation prior to vaccine formulation was not complete. There is good evidence that some FMD outbreaks probably had a vaccine origin. All these are powerful arguments to design vaccines that do not require infectious virus at any stage of their preparation. However, to achieve this goal a deep understanding of the molecular bases that govern biological and immunological properties of FMDV is necessary. The prediction of viral cross-protection remains an important unsolved problem, transcripts that can be blocked at some steps of virus replication or assembly, genetic complementation and molecular basis of virulence factors should all be deeply explored from the genomic knowledge.
- ii. Co-circulation of different types of FMDV is a reality in most parts of the endemic regions which represents a serious complication in the epidemiology of FMDV. Global epidemiological analysis is vital for implementing progressive regional foot-and-mouth disease control programs, but better knowledge of variability, recombination and evolution of FMDV is necessary. Development of spatial epidemic models to simulate transmission or to assess biosecurity planning and emergency-response preparedness requires better knowledge of FMDV evolution. Thus, to really understand FMD field epidemiology and how to contain the spreading of new outbreaks, wider molecular epidemiology analyses using full length genome information are necessary.

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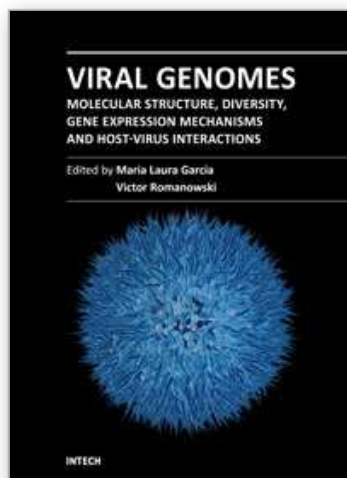
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