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Review article

**Foot-and-mouth disease virus:
a long known virus, but a current threat**

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Abstract – Foot-and-mouth disease virus (FMDV) was the first animal virus identified. Since then, FMDV has become a model system in animal virology and a considerable amount of information on its structure, biology and vaccinology has been obtained. However, the disease that this virus produces (FMD) still constitutes one of the main animal health concerns. In this review, we have attempted to summarise the state of the knowledge in different basic and applied areas of FMDV research, with emphasis on those aspects relevant to the control of the disease.

FMDV / structure / immunity / vaccine / variability / diagnosis

Résumé – Le virus de la fièvre aphteuse : un virus connu de longue date, qui demeure une menace. Le virus de la fièvre aphteuse a été le premier virus animal identifié. Depuis lors, il est devenu un système modèle en virologie animale, et une quantité importante d'informations sur sa structure, sa biologie et sa vaccinologie a été obtenue. Cependant la maladie provoquée par ce virus constitue encore une inquiétude majeure en santé animale. Dans cette revue, nous avons tenté de résumer l'état des connaissances dans différents domaines de recherche, à la fois fondamentaux et appliqués, sur le virus de la fièvre aphteuse, en mettant l'accent sur les aspects relatifs au contrôle de la maladie.

virus de la fièvre aphteuse / immunité / vaccin / variabilité / diagnostic

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Table of contents

1. The virus and the disease	2
2. Genomic organisation	4
3. The structure and antigenicity of the virion	7
3.1. Structure of the capsid	7
3.2. Antigenic structure	8
4. Cell recognition by FMDV	9
4.1. RGD-dependent mechanisms of cell recognition	9
4.2. RGD-independent mechanisms of cell recognition	9
4.3. Evolution of FMDV receptor usage and antigenicity	10
5. Immune response	10
6. Vaccines	12
6.1. Conventional vaccines	12
6.2. “Emergency” vaccines	13
6.3. Proteins, protein fragments and viral subunits	14
6.4. Peptide vaccines	14
6.5. Expression of viral proteins in replicating vectors	15
6.6. Genetically-engineered attenuated strains	16
6.7. DNA vaccines	16
7. Variability and evolution	16
7.1. Variability in cell culture	16
7.2. Serotype diversification	17
7.3. Phenotypic variability and antigenic evolution.....	18
8. Diagnosis and viral characterisation	19

1. THE VIRUS AND THE DISEASE

Foot-and-mouth disease (FMD) virus (FMDV) is the etiological agent of an important disease of livestock. FMD is highly contagious and affects artiodactylae, mostly cattle, swines, sheep and goats (reviewed in [11, 25, 36, 37, 79, 175]). The disease was first described in Venice in 1546 [96]. In 1898, Loeffler and Frosch [139] demonstrated that a filtrable infectious agent smaller than bacteria caused it; this was the first description of a virus producing an animal disease.

In spite of the considerable information accumulated in the last years on the virus, the disease and the availability of vaccines, FMD still affects extensive areas of the world [126], and is the most transmissible viral disease of animals [175]. FMD ranks first in the A list of infectious diseases of ani-

mals, according to the Office International des Epizooties [172]. Despite low mortality rates, FMD severely decreases livestock production and introduces important trade restrictions on animals and livestock products [175]. A recent example of the devastating consequences of FMD is the epizootic which occurred in 1997 in Taiwan, in which more than 4 million pigs had to be slaughtered [126]. Nowadays, as with many other infectious diseases, FMD distribution is clearly associated with areas with lower levels of development (Fig. 1) and it contributes to severe economic problems of many developing countries. FMD control in endemic areas is implemented by regular vaccination (see Sect. 6), which has resulted in the eradication of the disease in some areas of the world; examples are the European Union and, more recently, Uruguay, Argentina,

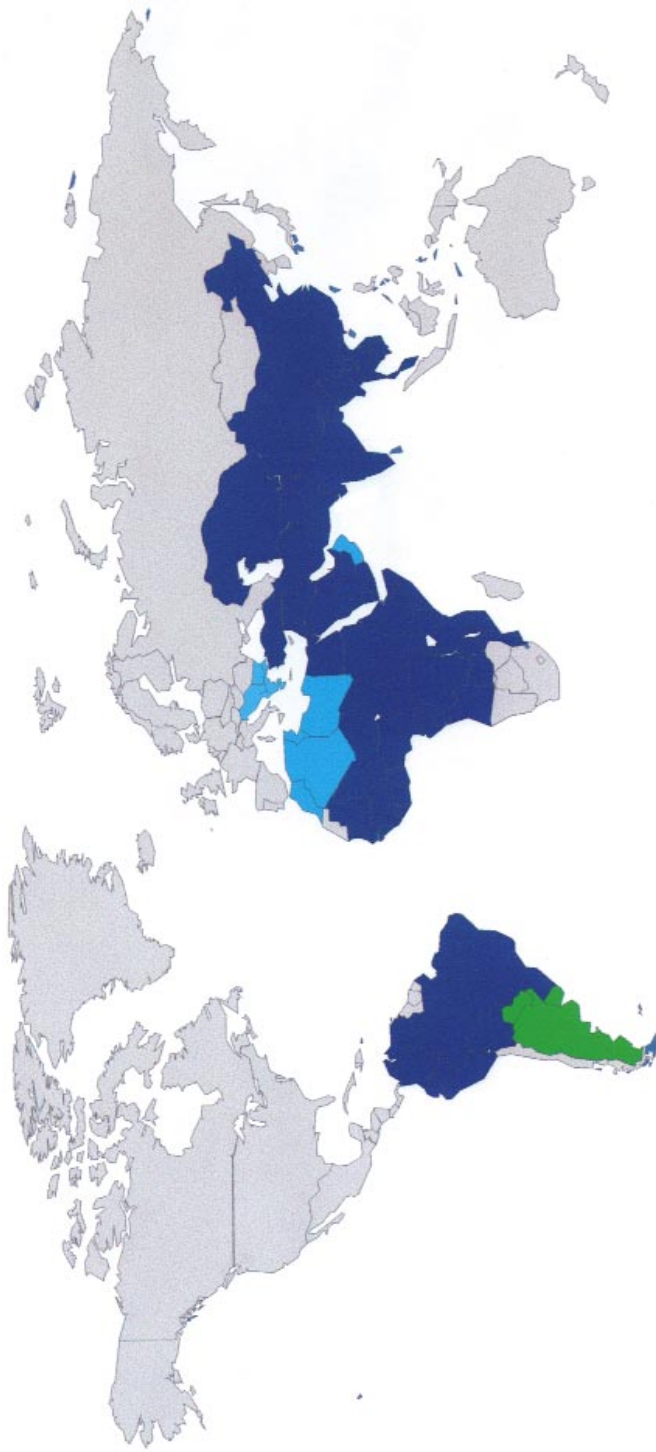


Figure 1. World distribution of FMD during 1997. ■ endemic area; ■ area with sporadic outbreaks; ■ area free of the disease but still vaccinating; ■ area free of the disease. Reprinted from Kitching R.P., A recent history of foot-and-mouth disease virus, J. Comp. Pathol. 118, pp. 89-108, Copyright 1998, by permission of the publisher WB Saunders.

Paraguay and the South of Brazil. A non-vaccination, stamping-out policy – implying slaughtering of infected and contact animals, together with animal movement restrictions –, associated with severe controls in the importation of animals from affected areas, have been shown effective in maintaining an area free of FMD [83]. However, the control of the disease is hampered by several socio-economic as well as technical factors; among the latter, the considerable antigenic diversity of the virus (see Sects. 6 and 7) precludes the preparation of a universal vaccine with unified quality control standards and worldwide distribution.

FMDV produces an acute, systemic vesicular disease. Besides farm animals, FMD also affects more than 30 wild ruminant species [224]. In natural infections, the main route of virus entry is the respiratory tract, and as few as one to ten infective particles can produce the disease [84, 220]. FMDV can be mechanically disseminated by animals, farmers, farming equipment, and during animal transport [36]. Long-distance, airborne transmission has also been documented [84, 124]. The initial virus multiplication usually takes place in the pharynx epithelium, producing primary vesicles, or “aphthae” [43]. The virus can also penetrate through skin lesions and it can be experimentally inoculated by intradermal injection into the tongue or in the claws. Within 24–48 h after epithelium infection, fever and viraemia start and the virus enters the blood stream and spreads to different organs and tissues, producing secondary vesicles preferentially in the mouth and feet. Little is known on the mechanism mediating the viral spread observed during the viraemia, although the involvement of macrophages in this process has been suggested [20, 251]. The acute phase of disease lasts about one week and declines gradually coinciding with the appearance of a strong humoral response [11]. In some cases, mortality can be observed among young animals, associated with lesions in the myocardium. The vesicles produced by FMDV generally affect cells

from the epithelial stratum espinosum [21, 251]. However, the ability of the virus to replicate in different internal organs and tissues, in which high virus titers are found [43], remains to be studied in detail. A high viral amplification frequently takes place in infected animals, being particularly dramatic in pigs, for which up to 10^{12} infectious units per infected animal have been scored [220].

In ruminants, an asymptomatic, persistent infection can be established [237] during which virus can be isolated from the oesophagus and throat fluids of the animals from a few weeks up to several years of the initial infection (reviewed in [208]). Both naive and vaccinated animals can become persistently infected [231] and they may do so following an acute infection. The mechanisms that mediate this persistence are unclear, but they are likely to result from a dynamic equilibrium between the host immune response and the selection of viral antigenic variants at the mucosae of the upper respiratory tract [103, 208]. There is epidemiological evidence to support that carrier animals may be the origin of outbreaks of acute disease, when brought into contact with susceptible animals [115]. However, to our knowledge, neither a definitive confirmation of this mode of transmission nor its possible epidemiological impact has been reported.

In vitro, FMDV productively infects several primary cultures as well as established cell lines such as BHK-21, IBRS-2 or BK cells. The intraperitoneal inoculation of FMDV produces death in suckling mice, and this has been extensively exploited to titrate virus infectivity. Likewise, FMDV can be adapted, by serial passages, to produce clinical symptoms in guinea-pigs, an animal model that has been used mostly for immunological analyses [98].

2. GENOMIC ORGANISATION

FMDV belongs to the aphthovirus genus of the *Picornaviridae* family [185, 197].

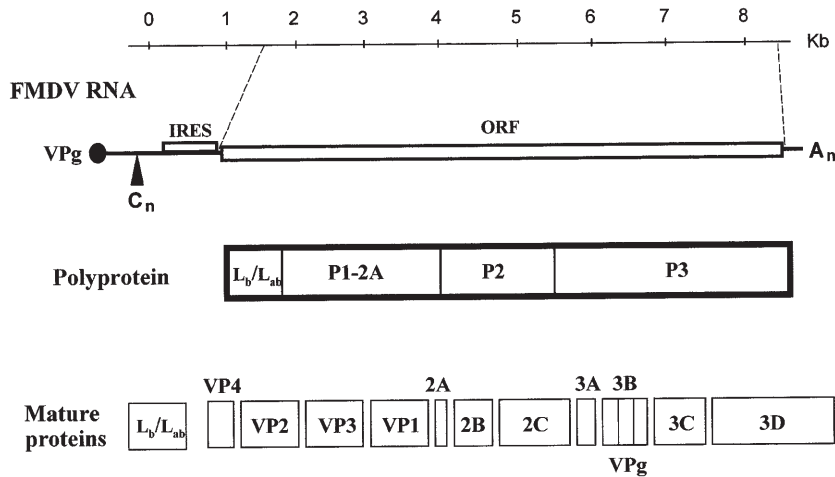


Figure 2. Scheme of the FMDV genome, the single encoded polyprotein (ORF) and the different viral mature proteins. The capsid proteins VP4, VP2, VP3 and VP1 are also termed 1A, 1B, 1C and 1D, respectively. VPg is the protein covalently linked to the 5' end. The non-coding regions (NCR) present at the 5' and the 3' of viral RNA, are indicated with a thick line. C_n points the poly C tract at the 5' NCR. The location of the internal ribosome entry site (IRES) is indicated. A_n denotes the poly A tail at the 5' end.

The viral particle contains a positive-strand RNA genome of about 8 500 nucleotides (nt) [11], enclosed within a protein capsid (see Sect. 3). The viral RNA consists of a single open reading frame (ORF), flanked by two non coding regions (NCR) (Fig. 2), both predicted to display complex secondary structures. Cis-acting structural elements involved in viral replication and gene expression are present in these NCRs. A small viral protein, VPg, is covalently linked to the 5' end of the molecule [213]. The 5'NCR (about 1 200 nt) is divided by a poly C tract located about 400 nt from the 5' end [41]. Little is known about the RNA region upstream of the poly C, except that a clover leaf structure is predicted at the 5' end [53]. A similar clover leaf structure found in poliovirus RNA is involved in the synthesis of positive strand RNA [7]. The poly C tract is present only in aphthoviruses and cardioviruses; its length varies among isolates

[41, 88] and it has been associated with viral virulence [114, 253] although definitive proof is still lacking. Highly structured pseudoknot motives, present in different number among isolates [88], whose function remains unknown, are predicted downstream of the poly C [53]. The translation initiation of the FMDV RNA starts at two AUG codons separated by 84 nt [23], following ribosome recognition of the upstream internal ribosome entry site (IRES), which spans the 465 nt preceding the first functional AUG [26, 132]. The picornavirus IRES element provides cap-independent translation function, as do those which are present in other viral RNAs and some eukaryotic RNAs [151]. This region is highly structured and contains several non-initiator AUG codons. Different RNA domains and functional features within this element, including the binding to cellular proteins, have been reported [141, 152, 164, 171]. A highly

ordered structure is also predicted at the 3'NCR of the FMDV genome and RNA transcripts, complementary to this region, transiently inhibit viral infectivity in cell culture [112]. This region, of about 90 nt, precedes a genetically encoded poly A tract [48]. There is extensive evidence of the interaction between the 3'NCR of picornaviruses and several viral and host proteins (reviewed in [3]).

Replication and translation of FMDV RNA [11, 212] occur in the cytoplasm of infected cells [8], and these biochemical processes are associated with cell membranes [29, 169]. FMDV RNA is infectious by itself, when transfected into susceptible cells [27]. This feature has made possible the construction of infectious cDNA clones [183, 253], and their use is a powerful tool to study different genes and functional motives of the viral RNA by the analysis of derivatives bearing mutations or deletions at preselected genomic sites (see Sects. 4 and 6).

The viral ORF encodes a single polyprotein, which is cleaved by viral proteases [201] to yield the different viral products (Fig. 2). The P1-2A region encodes the structural proteins VP1, VP2, VP3 (M.W. $27-30 \times 10^3$), and VP4 (M.W. $9-10 \times 10^3$). The 16-amino-acid peptide 2A catalyses in cis the excision of P1-2A from 2C [202]. The regions L, P2 and P3 encode eight different mature non-structural proteins (NSP). Each of them, as well as some of the processing intermediates, are involved in functions relevant to the virus life cycle in infected cells (reviewed in [25, 181]). The L region contains two overlapping proteins Lab and Lb, that result from the translation initiation from each of the two functional AUGs of the polyprotein [23, 214]. Lab and Lb catalyse their proteolytic excision from the polyprotein [228, 235] and initiate the cleavage of eIF-4F, a component of the cap-binding protein complex [67], leading to the shutoff of host protein synthesis in virus-infected cells. The L region is dispensable, since mutants lacking this gene

can replicate [178]. The roles of the polypeptides encoded in the P2 regions are poorly understood. Mutations that confer resistance to guanidine, an inhibitor of viral RNA replication, are located in 2C [219] and a helicase domain is present in this polypeptide [181]. In poliovirus, 2B and 2C are involved in viral RNA synthesis (reviewed in [109, 250]). The polypeptide 2C and its precursor 2BC are associated and induce cell membrane vesicle proliferation [29]. The P3 region comprises four mature proteins 3A, 3B, 3C and 3D. 3A has been proposed to be the membrane anchor for the picornavirus replication complex [250], it is associated to viral-induced membrane vesicles and contributes to the cytopathic effect and to the inhibition of protein secretion [71]. 3A is likely to play a relevant role in the pathogenesis of FMDV in natural hosts, since changes in 3A have been reported in several viral strains belonging to different serotypes and which are attenuated for cattle [21, 204]. The presence of three tandem, non-identical, copies of 3B (VPg) is unique among the picornaviruses [94, 123] representing a rare case of information redundancy in the genome of an RNA virus. VPg participates in the initiation of picornavirus RNA synthesis and a role in the encapsidation of viral RNA has also been proposed [250]. Viable viruses can be recovered from infectious RNAs carrying a single VPg copy, but the level of infectivity correlates with the number of copies present in the RNA [92]. In poliovirus, a protein complex including 3AB precursor and 3D interacts with the clover leaf structure present at the 5' NCR, as part of the process of plus strand RNA synthesis [250]. 3C is a thiol-protease responsible for most of the cleavage events undergone by the viral polyprotein [201, 235]. 3C also induces the proteolytic processing of histone H3 [91], which may relate to the inhibition of host transcription observed in infected cells. The 3D protein is the viral RNA-dependent RNA polymerase [169, 180].

3. THE STRUCTURE AND ANTIGENICITY OF THE VIRION

3.1. Structure of the capsid

Following cleavage of the structural precursor P1, three polypeptides, VP0 (precursor of VP4 and VP2), VP1 and VP3, assemble into asymmetric units or protomers. Five protomers associate, forming a pentamer, and twelve pentamers incorporate

rate a newly synthesised RNA molecule to form a virus particle (Fig. 3). Cleavage of VP0 to VP2 and VP4 is considered autocatalytic, and it is observed upon the encapsidation of RNA [62]. The structures of FMDV virions of serotypes O [2], C [135], and A [62], and some antigenic variants [136] have been elucidated by X-ray crystallography. FMDV capsids have the classic structural organisation of the picornavirus family. They consist of non-enveloped capsids of icosahedral symmetry, 28–30 nm in diameter, composed of 60 asymmetrical

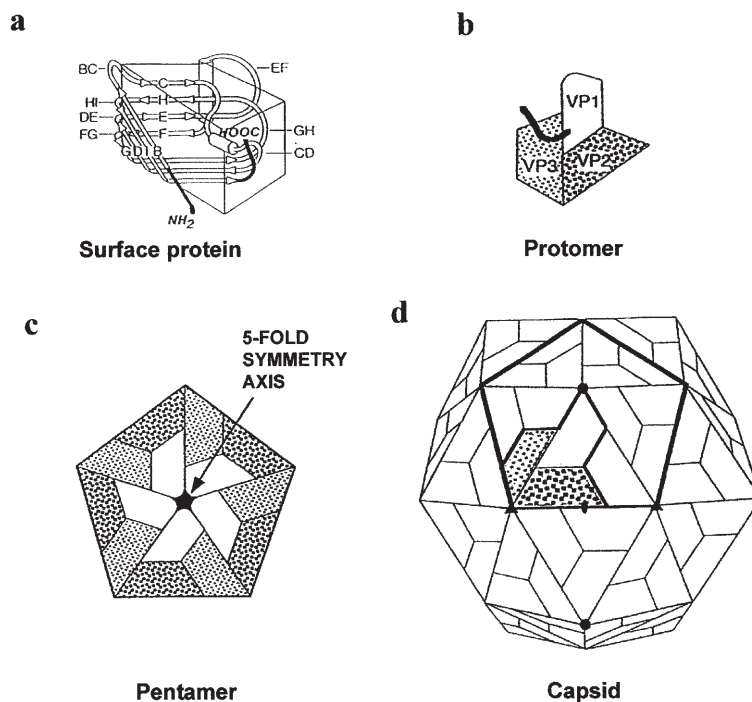


Figure 3. Schematic view of the structure of the surface FMDV capsid proteins, the subunits in which they assemble and the viral capsid. (a) Schematic, wedge shaped, tertiary structure of VP1, VP2 and VP3. The different structural elements are denoted as follows: β -chains, arrows; α -chains, cylinders; loops, ribbons; carboxi and amino terminal domains, black ribbons. The upper face of the trapezoid is exposed on the surface of the virion, and the opposite face is in contact with the RNA. (b) Arrangement of VP1-VP3 in a biological protomer; the location of the amino terminal domain of VP1 is indicated by a black ribbon. (c) Arrangement of five protomers into a pentamer. (d) Structure of the capsid; 2-fold, 3-fold and 5-fold axes are indicated, in black, by an ellipse, a triangle and a circle, respectively. A pentamer is outlined in the capsid, and a protomer is indicated inside the pentamer.

subunits, or protomers, each made up of one copy of four structural proteins, VP1-4 (Fig. 3). Amongst them, VP1, VP2 and VP3 are surface-oriented, whereas VP4 is internal and has a N-terminal myristic acid [52]. VP1-3 shares a similar structural pattern, consisting of an eight stranded β -barrel, composed of two four-stranded β -sheets. The loops joining these strands, as well as the C-termini of these three proteins, are exposed on the surface of the capsid, whereas their N-termini are located facing its interior. The antigenicity of these particles is usually associated with amino acid residues that are well exposed on the surface of the capsid (reviewed in [156, 234]).

Despite structural similarities among picornavirus genera, FMDV exhibits distinctive structural features. In most picornaviruses there is a prominent depression on the surface, termed “canyon”, which is involved in the interaction with the host cell receptor [116, 193]. In contrast, the FMDV capsid is relatively smooth with no obvious canyons or pits, but remarkably, the G-H loop of VP1 protrudes from the surface, and such a loop is the most prominent feature of the virion. The structure of this large loop has attracted considerable attention because of its mobility and multifunctionality. It contains a highly conserved Arg-Gly-Asp (RGD) motif that constitutes the main cell attachment site (see Sect. 4), and comprises a major neutralisation site (see below). The G-H loop spans about 20 residues around positions 140–160, and can adopt different conformations without apparent perturbations of the rest of the capsid. For this reason the electron density maps obtained from virions of serotypes O [2] and C [135], have not allowed derivation of a defined structure for this loop. In serotype O, in contrast to other FMDV serotypes, the base of the loop (Cys 134) is linked to VP2 (Cys 130) via a disulphide bond. Following chemical reduction of type O virions, the structure of this region becomes sufficiently ordered to allow the determination of a defined conformation [140]. The conformation of this

loop in serotype C was solved by the crystallographic analysis of a peptide that mimicked its sequence, and that formed a complex with a neutralising monoclonal antibody specific for this antigenic site [243]. The loop conformations thus determined for type O and type C are remarkably similar, in spite of low sequence identity. The conserved RGD motif adopts an open turn conformation between a β -sheet at its amino side and a helix at its carboxi side. Flanking these structural domains, residues lacking secondary structure are present that presumably confer flexibility to the loop, and connect it loosely to the capsid.

Unlike other picornaviruses FMDV capsids exhibit a highly hydrophobic hole at the 5-fold axis which allows penetration of molecules such as intercalating dyes and caesium ions [2]. This feature explains the inactivation of FMDV infectivity by photoreactive dyes and the high buoyant density of their virions ($1.43\text{--}1.45\text{ g}\cdot\text{cm}^{-3}$), the highest among the picornaviruses [11, 37]. FMDV particles are also unstable at $\text{pH} \leq 6$, and at physiological temperatures [11, 37].

3.2. Antigenic structure

A major continuous FMDV antigenic site is located in the G-H loop of VP1, as deduced from the immunogenicity of peptides spanning VP1 residues around positions 140 to 160 [30, 176, 229]. In addition, a large proportion of Mab resistant (MAR) mutants obtained with MAbs raised against entire viral particles include amino acid substitutions within this site (reviewed in [40, 156, 234]). For serotype C, the antigenic structure of the G-H loop is complex, since different overlapping epitopes, defined by their differential ability to react with individual MAbs, have been mapped within this loop [158, 159], reviewed in [156].

Two additional neutralisation sites have been described by analysing MAR mutants [17, 129, 135, 233]. Site C is located at the C-terminus of VP1 and is apparently

continuous and independent from the G-H loop in serotypes A and C [135]. In type O, its vicinity with the G-H loop in the structure of the capsid, as well as competition studies with neutralising MAbs, suggest that sites A and C conform a single site composed of discontinuous epitopes [17]. Site D is discontinuous, comprising residues involving all surface polypeptides, and lies within the C-terminus of VP1 (residue 193), the VP3 B-B knob (residue 58), and VP2 B-C loop (residues 72, 74 and 79) (positions are numbered, independently for each individual polypeptide, according to [135]). These antigenic sites are located at exposed regions adjacent to each other and close to the 3-fold axis of symmetry in the capsid.

4. CELL RECOGNITION BY FMDV

4.1. RGD-dependent mechanisms of cell recognition

Structural and functional studies with FMDV over the last decade have established that the G-H loop of capsid protein VP1 and, particularly, its RGD motif are critically involved in virus interaction with integrin $\alpha_v\beta_3$. The RGD sequence is a universal cell-recognition site present in various extracellular proteins that interact with cell surface integrin receptors [177]. The presence of a highly conserved RGD sequence within the extremely variable G-H loop region of FMDV capsid protein VP1 (reviewed in [79, 156]), together with the inhibition of virus adsorption by RGD-containing peptides [19, 95, 160], provided initial evidence of integrin molecules being involved in FMDV cell recognition and internalisation. Identification of integrin $\alpha_v\beta_3$ as the FMDV receptor came from studies with FMDV A12 on inhibition of cell adsorption and plaque formation by antibodies to $\alpha_v\beta_3$ [28], and more recently from the use of cell lines modified to express this integrin [167]. The requirement of an RGD-integrin binding motif for FMDV infectivity was confirmed

in serotypes A, C and O by direct mutagenesis of infectious cDNA clones of FMDV [14, 137, 154, 162], and FMDVs representative of each serotype were found to bind purified human integrin $\alpha_v\beta_3$ in an RGD-dependent manner [119]. Finally, recent evidence suggests that $\alpha_v\beta_3$ is a functional receptor for FMDV infections of cattle [167], yet the implication of other integrin molecules in the natural life-cycle of FMDV in this host remains to be determined [120, 121].

Binding affinity and integrin specificity of RGD-sites are affected by a number of structural parameters, and amino acid residues flanking the RGD-motif in positions -1, +1 and +2 have been shown to confer selectivity in synthetic integrin-binding peptides [177]. In serotype C, the use of synthetic peptides representing the G-H loop led to the identification of two highly conserved Leu residues located at position +1 and +4, and to a lesser extent the residue at position +2, as critical determinants in promoting cell recognition [160]. These particular amino acid residues have been found to be conserved in a large number of field isolates [160], and in MAR mutants of type C FMDV [150]. The influence of RGD-flanking residues on protein interaction with integrins suggests that subtle modifications at the G-H loop may have substantial implications for RGD-dependent cell recognition by FMDV [120].

4.2. RGD-independent mechanisms of cell recognition

Interaction with integrin receptor molecules is not required for FMDV uncoating, and the virus can infect cells by an antibody-dependent pathway [153, 154, 184]. This suggests that expression at the cell surface of particular molecules, which can act as receptors or coreceptors for the virus, may exert an important selective pressure on FMDV. Propagation of FMDV in cell culture led to the rapid selection of mutant

viruses which are characterised by multiple phenotypic alterations including improved replication capacity, enhanced resistance to neutralising MAbs, expanded cell tropism, binding to heparin and attenuation for cattle [14, 90, 118, 150, 203, 222]. These phenotypic traits were all found to be associated with a limited number of capsid alterations that confer to FMDV the capacity to use RGD-independent mechanisms of cell recognition [15, 167]. Binding to heparin, which has been associated with the capacity of FMDV to interact with heparan sulphate glycosaminoglycan residues on the cell surface [118, 203], involves acquisition of positively charged amino acid residues at the capsid surface [100, 203]. Interestingly, the location of these critical residues appears to vary for different FMDV strains or even for the same virus clone with different passage histories in cell culture [14, 90]. The recent identification of FMDV mutants which are able to enter cells via $\alpha_v\beta_3$ -independent and heparan sulphate-independent pathways supports the existence of at least an additional receptor molecule, and, thus, of multiple mechanisms for FMDV adaptation to cells in culture [15].

Interestingly, FMDV mutants, which displayed RGD-independent mechanisms of cell recognition, retained their capacity to interact with integrin receptor molecules [15]. This suggests a potential for these viruses to use multiple alternative receptors for entry even into the same cell type, as well as a capacity to modulate receptor usage in response to environmental modifications. Not only genomic changes in FMDV determine receptor usage, but also the same capsid may be driven to use one or another entry pathway.

4.3. Evolution of FMDV receptor usage and antigenicity

The capacity of FMDV to develop and to use multiple, alternative receptors for entry into the same cell type has implica-

tions for the evolution of virus antigenicity. Structural studies performed with an antigenic peptide representing the G-H loop of VP1 have indicated that the RGD motif, and in particular the Asp residue, is not only part of the receptor recognition site but it also interacts directly with some anti-viral neutralising antibodies [243, 244]. This shows that amino acid residues which play a critical role in receptor recognition can also be involved in interactions with neutralising antibodies, and it is the negative selection of variants which allows the conservation of such residues in FMDV when they are required for virus infectivity [156]. In contrast, MAR mutants with an alteration at the RGD motif can be selected from FMDV populations, which acquired RGD-independent mechanisms of cell recognition [15, 150]. Analysis of the antigenic properties of a multiple passaged FMDV clone with an RGG sequence instead of the RGD motif confirmed that large antigenic variations can be prompted by replacements at capsid residues involved in receptor recognition [198]. Although the replacement of Asp143 by Gly within the RGD motif of the G-H loop abolished FMDV interaction with its integrin receptor molecule [15], RGG-containing FMDV replicates efficiently in cell culture and is genetically stable in a large proportion of serial infections [150, 198]. Evolution of FMDV in cell culture can reduce the constraints in an important antigenic site involved in integrin-receptor recognition, and may allow the virus to explore new antigenic structures.

5. IMMUNE RESPONSE

Upon infection, FMDV elicits a rapid and broad spectrum of immune mechanisms, including humoral and cellular responses (see Fig. 4) that induce an efficient protection against reinfection with homologous and antigenically related viruses (reviewed in [55, 161, 208]). Neutralising antibodies directed to well characterised B cell epitopes

located on the viral capsid (see Sect. 3) can be detected soon after infection or vaccination with FMDV. The first neutralising antibodies, that appear as early as 3 to 4 days

following infection or vaccination, are IgMs; in cattle this response peaks around 10 to 14 days post-infection, and then declines [55]. IgGs are detected in pigs

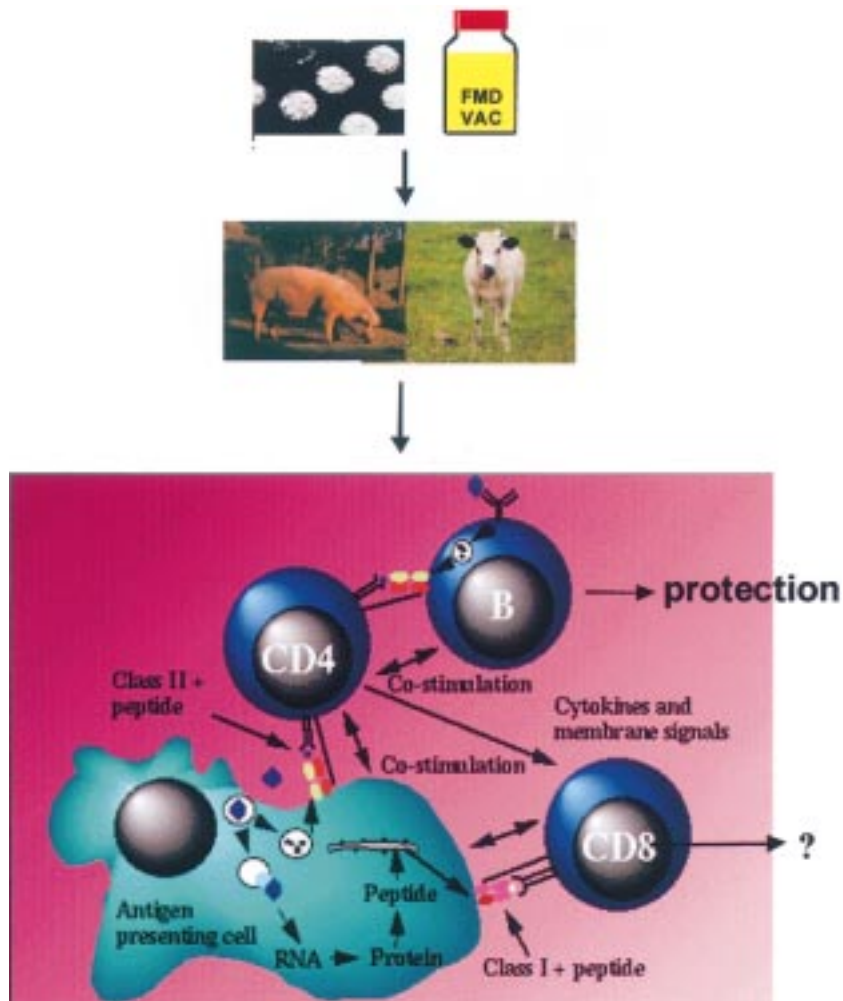


Figure 4. Schematic representation of different branches of the adaptive immune response induced by FMDV. Viral peptides (T cell epitopes) are presented, associated with MHC class II molecules, to CD4 T lymphocytes on the surface of antigen presenting cells. Activated CD4 T cells can cooperate to an efficient production of anti-viral antibodies by B lymphocytes (associated with protection to FMD), previously activated by its interaction with B cell epitopes present on viral proteins. Immune CD4 cells can also interact with CD8 T lymphocytes, previously activated by its interaction with viral peptides presented, in association with MHC class I molecules, on the surface of infected cells, to trigger a cytotoxic (CTL) response. The role of the CTL response in FMD protection is not well established and this is indicated by a question mark.

between 4 to 7 days post-infection and become the major neutralising antibodies by two weeks post immunisation [97]. In both, infected and vaccinated cattle, IgG1 response is generally greater than that of IgG2 [166]. Early upon infection or vaccination, there is a detectable antibody response in the secretions of the upper respiratory and gastrointestinal tracts [97]. The major antibody subclasses found in secretions are initially IgM and then followed by IgA and IgG (reviewed in [208]). However, little is known on the contribution to the protection of innate immune responses in FMDV immunised animals.

Protection against FMD is often associated with the induction of high levels of neutralising antibodies in serum [236]. However, this response does not ensure clinical protection and animals with low serum neutralisation (SN) titers may nevertheless be protected [55, 161]. It has been proposed that phagocytosis of virus-antibody complexes, following viral opsonisation, may mediate viral clearance in vivo [161]. Due to the early evidence of involvement of antibodies in protection against FMD, immunological studies have been mainly focused on the humoral response elicited by FMDV. However, as supported by recent findings, T cell responses also contribute to the immunity to this virus (reviewed in [24, 55]). In mice, FMDV antibody responses are T cell dependent [57], even when the induction of a T-cell independent response has been reported in animals immunised with high doses of virus [33]. In cattle and pigs, B cell activation and antibody production are associated with a lymphoproliferative response mainly mediated by T cells (mostly CD4⁺). These T helper cells recognise a number of viral epitopes, located both in capsid proteins [31, 56, 58, 102, 189, 205, 238, 240] and NSP [59]. Thus, it appears that the T cell responses mediated by CD4⁺ cells are required for protective immunity against FMDV, by participating in the production of antiviral antibodies [31, 58, 240] and by maintaining the appropriate microenviron-

ment needed for a synergistic immune response. The induction of FMDV-specific effector cytotoxic T lymphocytes (CTL) has been difficult to evaluate [5, 50, 190, 215]. Recent studies have shown that FMDV infection results in a rapid reduction of MHC class I expression on susceptible cells [216]. Among the consequences of this effect is the impairment of the presentation of viral peptides by FMDV infected cells to CTLs, and this may facilitate virus escape from this particular antiviral response of the host.

6. VACCINES

6.1. Conventional vaccines

Conventional vaccines against FMD are based on chemically inactivated viruses [18]. Formalin was the first inactivant used for their production; however, its association with the emergence of viral escapes [22] led to the use of binary ethyleneimine as an inactivant [13]. The effective formulation of FMD inactivated vaccines requires adjuvants, and Al(OH)₃/saponin (for ruminants) and incomplete oil-based formulations (for pig and cattle) have been widely employed (reviewed in [72]). Vaccine formulations frequently include viruses of different serotypes, and vaccine preparation implies the growth of high quantities of virus in tissue culture; therefore, high-containment facilities are required. The use of high quality vaccines, with an antigenic composition adapted to the circulating viruses, has decisively contributed to the eradication of FMD in several areas of the world (see Sect. 1). Inactivated vaccines elicit a consistent humoral response, albeit generally weaker than that induced in infected animals [18]. This response correlates with a solid lymphoproliferative response [56, 102, 205], and with considerable, although short-lived, protection [18, 72]. However, the spectrum of antiviral immune mechanisms induced by inactivated vaccines is limited [18,72]. Furthermore, they do not prevent the

persistent infection of protected ruminants with FMDV [208].

6.2. “Emergency” vaccines

In the last two decades many FMD-free countries have gradually moved toward the holding of strategic reserves of foot-and-mouth disease vaccines in the event of an outbreak. Whilst these countries rely primarily on slaughter, movement restrictions and zoosanitary measures for control, such vaccines would offer supportive measures in case an outbreak becomes more extensive.

Reserves, or banks, consist of either ready formulated vaccines, which have to be replaced every 12–18 months, or the more economical and popular method, storing concentrated antigens indefinitely over liquid nitrogen, which can be rapidly formu-

lated into vaccine in the event of an outbreak. Good examples of the latter include the North American Vaccine Bank, the International Vaccine Bank and the European Union Vaccine Bank and there are also numerous examples of similar National Banks. Unlike conventional FMD vaccines, however, emergency vaccines are often of higher potency (≥ 6 protective dose 50 (PD50)) to ensure both rapid protective immunity and greater cross-specificity. The high potency is usually the result of increasing the antigen load per dose and there is good documentary evidence that such emergency vaccines, as either an oil or aqueous formulation, confer rapid and protective immunity in the appropriate target species within 4 days of vaccination [61, 74, 209]. Additionally, such vaccines also appear to be able to reduce local virus replication in the oropharynx thereby limiting the transmission

Table I. Characteristics of different FMD vaccines.

	Conventional inactivated vaccines	Emergency vaccines	Viral subunits and synthetic peptides	DNA vaccines and recombinant viral vectors	Bio-engineered attenuated viruses
Immunogenicity	High	High	Limited, except for empty capsids	Potentially high	High
Thermal stability^a	Low	Low	High, except for empty capsids	Potentially high for DNA vaccines, low for viral vectors	Low
Innocuousness and safety in the production	Limited	Limited	High	Not studied	Not studied
Duration of immunity	Limited	Improved	Not studied	Not studied	Not studied
Spectrum of protection	Limited	Improved	Not studied	Not studied	Not studied
Distinction between infected and vaccinated animals	Limited	Limited	Good	Good	Potentially good

^a The thermal instability of FMDV virions makes a cold-chain necessary to preserve and store commercial vaccines.

of disease to other susceptible animals [61, 209]. More recently, studies using an oil-based high potency vaccine in sheep [16] have shown that after only a single vaccination, the antibody levels can be maintained for a significantly longer period of time than normally observed with a conventional formulation. Therefore, there is little doubt that the use of high potency formulations can provide additional benefits to those associated with the conventional vaccine.

Table I summarises the limitations of conventional vaccines and the different approaches that are being used to develop alternative safe and effective vaccines. As discussed below, although much has been learned from such research, it is increasingly clear that further understanding of the correlates of protection against FMD, and of mechanisms of immune evasion by the virus are required before effective new vaccines can be designed.

6.3. Proteins, protein fragments and viral subunits

Early observations indicated that isolated VP1, and fragments derived from its carboxyterminal-half, were the only viral capsid products capable of inducing neutralising antibodies and conferring partial protection [12, 134, 163, 229]. Thus, most attempts to develop synthetic vaccines were made using the entire VP1 or some of its fragments (reviewed in [38, 39, 49, 79]). Initial experiments indicated that immunisation with bacterially produced VP1 conferred protection in the pig [130]. However, the immunogenicity of VP1 produced in microorganisms is several orders of magnitude lower than that of the equivalent amount of antigen incorporated in viral particles [38, 39, 79]. This lower immunogenicity may be related to an inadequate folding adopted by VP1 in solution, which would limit the exposure of immunogenic sites to the host immune system. Recently,

high levels of neutralising antibodies were reported in mice immunised with VP1 obtained from transgenic plants [47].

Empty capsids retain most of the immunogenic and antigenic properties of viral particles [111, 195] and therefore, their production in recombinant vectors for vaccine purposes has focused the attention of different groups. The P1 polyprotein expressed in baculovirus retains the antigenicity of both continuous and discontinuous B cell antigenic sites present in the viral capsid [1, 207]. Immunisation of pigs with P1 produced in *E. coli* resulted in the induction of protection [111]. However, the expression of P1 polypeptide in vaccinia and baculovirus have yielded limited amounts of empty viral capsids [1, 138, 192, 207], whose production requires adequate P1 processing provided by 3C [110, 138]. Therefore, further work is required to improve the efficiency of empty capsid formation.

6.4. Peptide vaccines

The large amount of information available on the antigenic structure of this virus (see Sect. 3) has allowed the design of vaccines based on synthetic peptides corresponding to B cell epitopes identified on the viral capsid. Most of the work has been done using a continuous, immunodominant B cell site located in the G-H loop around positions 140–160 of capsid protein VP1 (see Sect. 3, reviewed in [38, 39, 55, 79, 156]). Uncoupled peptides containing the G-H loop of VP1 from viruses of different serotypes induced neutralising antibodies in guinea pigs and mice [99, 176] and protection in swine [30]. Among the different attempts to improve the immunogenicity of these peptides, its expression as part of the hepatitis B core structure was the most promising [54]. In 1986, DiMarchi et al. [70] reported protection to viral challenge in cattle immunised with a peptide in which the amino acids of the G-H loop were

co-linearly synthesised with those corresponding to the carboxy terminus of VP1 (residues 200–213). The induction of significant levels of heterologous protection in the guinea-pig with this tandem peptide has also been reported [73]. However, the immunogenicity of these peptide constructs in a number of host species was substantially lower than that of conventional vaccines (reviewed in [55, 79]). In the following years, the concept that immunogenic peptides should also include viral T cell epitopes to provide an adequate co-operation with immune B lymphocytes to induce an effective neutralising antibody response, became generally accepted [39, 55, 58, 79, 98, 194, 227]. Such T cell peptides should ideally be recognised by T lymphocytes in the context of alleles of MHC class II frequently represented in the populations of the natural hosts [194]. Even when G-H loop peptides can stimulate T cells of individuals from host species [105, 189, 232, 252], this recognition seems to be considerably restricted by the individual MHC composition [101, 104–106, 239], and may vary depending on the sequence of the viral peptide used [105]. Immunisation with peptides including the G-H loop either alone or in combination with an independent T cell epitope has been reported recently [232], using a large number of cattle. The different peptides induced partial protection to viral infection that ranged from 23 to 39% of animals being protected. A high animal to animal variation was observed in both the *in vitro* SN titers and the virus-specific T cells elicited. Consistently with the need of efficient T cell epitopes, the induction of T cell response was associated with protection. Furthermore, FMDV mutants with amino acid substitutions at the G-H loop were found in 41% of the lesions developed by peptide-vaccinated animals. This result supports that the high potential for antigenic variation exhibited by FMDV underlies the selection of antigenic variants in animals vaccinated with simple immunogens, such as peptides (see Sect. 7).

Improvement of the B cell epitopes to be included in peptide vaccines may require the synthesis of complex peptides able to mimic discontinuous epitopes relevant to virus neutralisation (see Sect. 3). Retro-inverso peptide analogues, with modifications in the amide bond, are more resistant to protease degradation [241], and have been used to enhance the immunogenicity of FMDV antigenic peptide vaccines, with promising results in mice and guinea-pigs [34]. The use of mixtures of peptides covering different antigenic variants, termed mixotopes [108], may decrease the chances of selection of escape mutants. New immunodominant T cell epitopes in FMDV structural and non-structural proteins [31, 240] could be included in the design of peptide vaccines in an attempt to overcome the restriction introduced by the MHC polymorphism in their recognition by T cells. It is hoped that the application of a combination of these new developments will result in the design of safer and more effective anti-FMD peptide vaccines.

6.5. Expression of viral proteins in replicating vectors

A more efficient induction of protective immunity by live vaccines, as compared to inactivated antigens, has been reported for a number of viruses, including some of the picornaviridae family [234]. This is expected from a requirement for a broad spectrum of immune responses to achieve an efficient protection, as observed in animals following natural infection. One strategy to achieve this goal is to present foreign antigens in a replicative form, expressed from recombinant viral vectors. It has been shown that recombinant adenovirus expressing P1 confers partial protection against FMDV challenge in cattle [218] and pigs [217]. In contrast to that which is often observed with inactivated vaccines, the protection observed with recombinant adenovirus expressing P1 was elicited in the absence of detectable

anti-viral antibodies, and it is likely to be mediated mainly by cellular immune responses [217].

6.6. Genetically-engineered attenuated strains

Dangers inherent to the high potential for variation and adaptation exhibited by FMDV (see Sect. 7) has hampered the use of classical attenuated strains, obtained by the adaptation and further passages of virulent viruses in nonsusceptible hosts [204], as vaccines. This is due to the frequent reversion of attenuated viruses to virulent forms [44], as well as the fact that viral strains attenuated for a given host may be virulent for other natural hosts [204]. New attenuated virus vaccines have been designed by modification of infectious FMDV clones. Chimeric viruses in which the RGD receptor binding site [162] or the L gene [155] were deleted, induced protection in natural hosts without producing clinical symptoms. In spite of these promising results, the wide FMDV host-range and the high potential for variation of the virus make a careful study of the stability and pathogenicity of new recombinant vaccines necessary before they can be considered for field trials.

6.7. DNA vaccines

Immunisation with naked DNA can elicit humoral and cellular immune responses and protection against different pathogens [249]. The induction of protection in swines immunised with a DNA vaccine containing an FMDV attenuated full length infectious clone, has been recently reported [247]. In this attenuated virus, the L gene and the RGD receptor-binding site were deleted. A neutralising antibody response has been observed in mice immunised with a DNA vaccine expressing empty capsids [51]. The effectiveness of DNA vaccines may be potentiated by the co-expression of FMDV

immunogens and cytokines relevant to the induction of protective immunity [133].

7. VARIABILITY AND EVOLUTION

7.1. Variability in cell culture

FMDV has been one of the model systems for the study of the high potential for variation of RNA viruses (reviewed in [79–81]). The studies made by Domingo et al. [78], mainly working with FMDV, and Holland et al. [117] using the vesicular stomatitis virus (VSV) as model systems, have provided important experimental and conceptual support to the notion that populations of RNA viruses consist of multiple variants collectively termed quasispecies [87]. In the quasispecies, a complex equilibrium between a high mutation rate, inherent to RNA replication, and the competitive fitness in response to the selective pressures, takes place continuously [76, 78]. This property endows RNA viruses with a high potential for virus variation and adaptation [76, 117]. In this section attention will be focused on commenting on those features that, in our opinion, are more relevant for FMD control.

Genetic and antigenic heterogeneity of FMDV populations, as well as high rates of fixation of mutations, have been observed in populations derived from cloned viruses upon a limited number of acute or persistent infections in cell culture [225]. The antigenic heterogeneity of FMDV populations was revealed by the high frequency of isolation (around 2×10^{-5}) of MAR mutants, estimated after minimal amplification of cloned viruses [148]. Propagation of these heterogeneous populations can result in a rapid emergence of antigenic variants even in the absence of immune pressure [32, 68, 81]. Such an emergence may constitute a problem for vaccine efficacy, since serial passages of vaccine and challenge strains are usually required for vaccine

production and testing [107]. The analysis of viruses recovered from serial passages of BHK-21 cells persistently infected with FMDV demonstrated a gradual accumulation of nucleotide substitutions [65] and the rapid generation of heterogeneity and the occurrence of phenotypic changes [66, 69]. A coevolution of both, viruses (which showed increased virulence for the parental BHK-21 cells) and cells (which became progressively resistant to the initial virus) was documented in great detail [66]. Likewise, the analysis of the viral populations obtained after multiple passages in BHK-21 cells allowed the exploration of the limits of the adaptation of the virus to a constant cell culture environment (see Sect. 4) and to document that the viral population size used in serial infections may influence viral fitness [89] and the type of variants that become dominant [221].

The potential for variation of FMDV has also been observed in host animals. Sequence heterogeneity among individual cloned viruses recovered from a single animal has been documented [77, 124, 196]. Genetic and phenotypic heterogeneity has been found in viral populations recovered upon infection of swine with plaque-purified viruses [45, 46]. Likewise, an analysis of long-term, persistently infected cattle, revealed the heterogeneity of the viral populations recovered [103]. In these animals, rates of fixation of mutations as high as 0.9×10^{-2} to 7.4×10^{-2} substitutions per site and year (s/s and year) were found in the VP1 gene [103]. High rates of fixation of mutations in VP1 and other structural genes [226], as well as the selection of antigenic and immunogenic variants [147, 157, 159] have also been reported during field outbreaks.

Recently, model studies with FMDV in cell culture have documented that viral quasispecies may possess a memory of their past evolutionary history in the form of minority components of the mutant spectrum [199]. This observation may be relevant to the response of RNA viruses to fluctuating selective constraints during prolonged infections in vivo [75].

7.2. Serotype diversification

During the centuries of evolution of FMDV in the field, repeated opportunities for variation have led to the viral diversification which is observed nowadays, reflected in seven serotypes which co-exist in the world (see Sect. 8). The availability of a considerable number of VP1 RNA sequences has allowed the derivation of representative phylogenetic trees relating multiple isolates of the seven serotypes [85, 86]. The trees show a reasonable correlation between classical serological groupings (serotypes) and well-defined genetic groups. Assuming an overall constancy in the FMDV evolutionary rate [149], an early lineage diversification was identified (Fig. 5), which gave rise to the three South African serotypes (SAT1, SAT2 and SAT3) as well as to a lineage which was the precursor of the present day A, C, O and Asia 1 serotypes. A further diversification which produced the different Euro Asiatic serotypes (A, C, O and Asia 1) circulating at present, seems to have occurred more recently [86]. The genetic diversification within serotype C was studied in detail over six decades [149] and has shown six major evolutionary lineages, that largely correlate with the geographical origin of the viruses, and a complex network of sublines in most of them. As previously reported by Beck and Strohmaier [22], European isolates are the exception, since they are considerably more homogeneous and clearly associated to vaccine strains. A similar evolutionary trend has been reported for type A and O viruses [9, 206]. The molecular basis of the serotype diversification is not fully understood and may have involved both positive selection and random sampling events (reviewed in [79]). We favour the view that the differences among serotypes are likely to have originated from selection, mostly due to

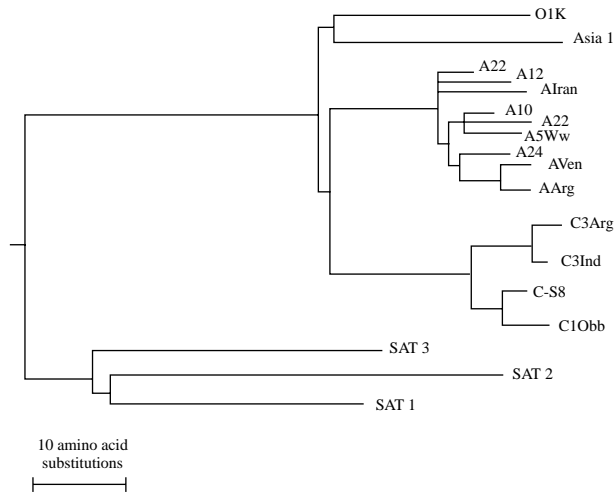


Figure 5. Phylogenetic tree relating isolates from the seven FMDV serotypes. The tree, adapted from [86], is based on the analysis of VP1 amino acid sequences.

immune pressure, acting on different antigenic motifs on the viral capsid when adaptive changes were compatible with the preservation of the basic architecture and the essential properties of the virion [82].

The most frequent mutational events observed during FMDV evolution are point mutations. Insertions and deletions appear to be fixed at lower frequencies. In vitro recombination in RNA viruses was first described with FMDV [125], and it occurs at high frequency among highly homologous strains in cell culture (reviewed in [122]). The limited evidence of FMDV recombination in the field [131] may reflect the low probability of co-infection of animals with unrelated FMDV strains.

7.3. Phenotypic variability and antigenic evolution

The analysis of RNA sequences from field isolates indicates that fixation of mutations occurs along the entire FMDV genome, including the ORF and the NCRs.

However, mutations are preferentially accumulated in genomic regions, which are the target of selective pressures [146]. In particular, capsid protein VP1 greatly contributes to the antigenicity of FMDV (see Sect. 3) and, therefore, its variations have been studied in detail (reviewed in [79, 156]). Extensive antigenic variation of FMDV type C in the field has been documented using MAbs specific for the G-H loop of VP1 [157, 158], and specific for the discontinuous site D [93] (see also Sect. 3.2). The number of variant sequences found was so high that this led to the proposal that individual FMDV isolates are not only genetically unique but are also antigenically unique in terms of their epitope composition [157].

Two mechanisms of diversification of the major antigenic site at the G-H loop of FMDV type C VP1 have been distinguished. The most frequent mechanism involves a gradual accumulation of amino acid replacements, equivalent to the classical antigenic drift, well known for human influenza virus type A [248]. Alternatively, a second

mechanism consists in an abrupt antigenic change due to single, critical amino acid substitutions that affect multiple epitopes, which would be conceptually parallel to an antigenic shift in the influenza virus type 1, but mediated by point mutations. These major antigenic changes have been documented both in field isolates and among laboratory-derived MAR mutants [159]. Substitutions in VP1 occur preferentially at a limited number of sites exposed on the virion surface [86, 149]. Moreover, the evolution of serotype C during sixty years occurred without a net accumulation of amino acid substitutions with time, in spite of accumulation of synonymous nucleotide replacements [149]. These observations suggest that important constraints (probably structural requirements) may limit VP1 variation in the field [82, 86].

The FMDV potential for variation in polypeptides other than those included in the capsid may also affect important biological properties such as viral virulence. The type O viruses isolated during the Taiwan 1997 epizootic showed high virulence for pig and low virulence for cattle. The reduced virulence for cattle has been associated with genetic alterations in NSP 3A, probably with the participation of other genomic regions [21]. In addition, the analysis of a guinea-pig adapted type C isolate has revealed that a single amino acid substitution in 3A mediates the ability to produce clinical symptoms in this host (Núñez et al., manuscript in preparation).

8. DIAGNOSIS AND VIRAL CHARACTERISATION

FMD requires a differential diagnosis with respect to other symptomatologically related diseases such as those caused by swine vesicular disease virus (SVDV) or VSV [230]. In addition, the antigenic variation exhibited by FMDV has largely conditioned the strategies followed for its diagnosis, both using genomic and serological

procedures. Seven serotypes of FMDV (A, O, C, Asia1, SAT 1, SAT 2 and SAT 3) have been identified on the basis of the ability of viruses to induce cross-protection in animals (reviewed in [174]). This cross-protection is serotype-restricted, and it is not always complete when tests involve different subtypes and variants of the same serotype [175]. Within serotypes, numerous subtypes can be identified depending on their antigenic similarity [35, 128]. However, the antigenic diversity of the virus is so complex that the initial classification into sub-types was considered as artificial and confusing [10, 200] and was finally replaced by the characterisation of virus reactivity against a panel of reference sera [127]. Classical techniques, such as complement fixation (CF) tests or SN tests using established cell lines such as BHK-21 or IBRS-2, have been and are still in use for the routine detection of the virus in clinical samples [172]. These assays are mostly based on the detection of capsid proteins, those that allow serological differentiation between serotypes. More recently, ELISA alternatives have been developed to identify and type FMDV isolates [191]. In general, these techniques are based on the use of serotype-specific antisera. In spite of a considerable library of type-specific MAbs available in different laboratories, such reagents have not been incorporated into routine detection procedures, due to the high diversity, at the level of single epitopes, shown by the virus. In most cases, growth in tissue culture of virus from field samples is required to obtain antigen for these analyses.

The serological detection of FMDV infection has been carried out by CF, SN and ELISA [113]. With the three methods, the detection is serotype-specific and does not allow a reliable distinction between infected and vaccinated animals [179]. Distinguishing infected from vaccinated animals is important, particularly for cattle, since they frequently develop a persistent and inapparent infection, even among vaccinated animals (see Sect. 1). The specific

diagnosis of infection has been performed using the classical virus infection associated (VIA) antigen, now known to correspond to the 3D polypeptide. The standard assay involves the agar gel immunodiffusion test [60]. Infection can also be ascertained by virus isolation from animal tissues or secretions [42]. To overcome the low sensitivity of these procedures, specific ELISA assays to detect antibodies against VIA antigen [4] and 3D expressed in *E. coli* [245] have been developed. However, positive serology to these antigens has been reported, mostly in re-vaccinated animals, since commercial vaccines produced from extracts of virus infected cells usually contain sufficient 3D protein for the latter to be immunogenic [179]. The association of small amounts of 3D with purified FMDV virions has been reported [170] and may contribute to the immunogenicity of 3D in vaccinated animals. The availability of other NSP, expressed in different systems, has recently allowed identification of new serological markers of FMDV infection [142, 143, 168, 187]. In particular, promising results have been reported with ELISA based on the detection of 3AB-3ABC antibodies [64, 187, 223].

The advent of RT-PCR procedures has led to the development of several RT-PCR assays for the specific detection and typing of FMDV RNA [6, 165, 172, 182, 186, 188, 242]. These procedures are highly sensitive and reduce the time needed for viral detection. In addition, RT-PCR amplification, in combination with direct nucleotide sequencing, has become an important tool for the rapid characterisation of field isolates and the tracing of new outbreaks [9, 128]. Sequencing analyses have been mainly focused on the gene encoding capsid protein VP1 (see Sect. 7). For practical reasons, VP1 sequencing has been limited to the 200–250 nt from the 3' end of VP1. This region contains the highly variable antigenic site around the G-H loop (see Sect. 3) and it allows derivation of informative phylogenetic comparisons [145]. Its analysis has

provided a considerable amount of information for type O, A, C, Asia, SAT 1 and SAT 2 serotypes [63, 144, 173, 210, 211, 246]. The OIE/FAO World Reference Laboratory for foot-and-mouth disease, at Pirbright, UK edits a web page in which an FMDV sequence data base is available (<http://www.iah.bbsrc.ac.uk/virus/Picornaviridae/aphthovirus/fmdv.htm>). It would be highly desirable to extend sequencing to the entire capsid-coding region of new FMDV isolates on a routine basis, since much information would be gained for adequate typing and for vaccine development. FMD remains a serious economic problem for the weakest countries of the world, and the technologically more advanced countries should facilitate expertise to control this dreaded disease.

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