Virus Adaptation and Treatment

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REVIEW

Use of recombinant capsid proteins in the development of a vaccine against the foot-and-mouth disease virus

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Abstract: Foot-and-mouth disease remains one of the world's most economically important diseases of livestock. It is caused by foot-and-mouth disease virus, a member of the picornavirus family. The virus replicates very rapidly and can be efficiently transmitted between hosts by a variety of routes. The disease has been effectively controlled in some parts of the world but remains endemic in many others, thus there is a constant risk of introduction of the disease into areas that are normally free of foot-and-mouth disease with potentially huge economic consequences. To reduce the need for large-scale culling of infected, and potentially infected, animals there has been significant effort to develop new vaccines against this disease which avoid some, or all, of the deficiencies of current vaccines. A major focus has been on the use of systems that express the structural proteins of the virus that self-assemble to generate "empty capsid" particles which share many features with the intact virus but lack the ribonucleic acid genome and are therefore non-infectious. Such particles can be "designed" to improve their stability or modify their antigenicity and can be produced without "high containment" facilities. The development and use of such improved vaccines should assist in the global efforts to control this important disease.

Keywords: picornavirus, diagnostic assays, virus structure, infection, immune responses

Introduction

Foot-and-mouth disease (FMD) is one of the most-feared diseases of farmed animals. It affects over 100 countries globally and many of the countries in Africa, the Middle East, and southern Asia experience FMD as an endemic disease. The disease continues to cause huge economic losses (estimated at around US$10,000,000,000 per year) on a global basis[1] and can cause losses of this magnitude in a single country when incursions occur into a state that is normally FMD free (eg, in the UK in 2001). The latter outbreak affected over 2,000 premises and resulted in the slaughter of several million animals. Many of these animals were not actually infected but were culled to prevent further spread of the disease or for welfare reasons. The mass culling of animals is clearly undesirable and has generated renewed interest in the development of efficient and safe vaccines that can be used to assist in the control of FMD outbreaks. The economic impact of FMD means that there are large barriers to prevent trade in animals, and their products, from areas of the world with FMD.

FMD is caused by infection with foot-and-mouth disease virus (FMDV); this is the prototypic member of the *Aphthovirus* genus within the family *Picornaviridae*. As with all picornaviruses, FMDV has a positive-sense, single-stranded, ribonucleic acid (RNA) genome (Figure 1). Within the virus particle, a single copy of the genome

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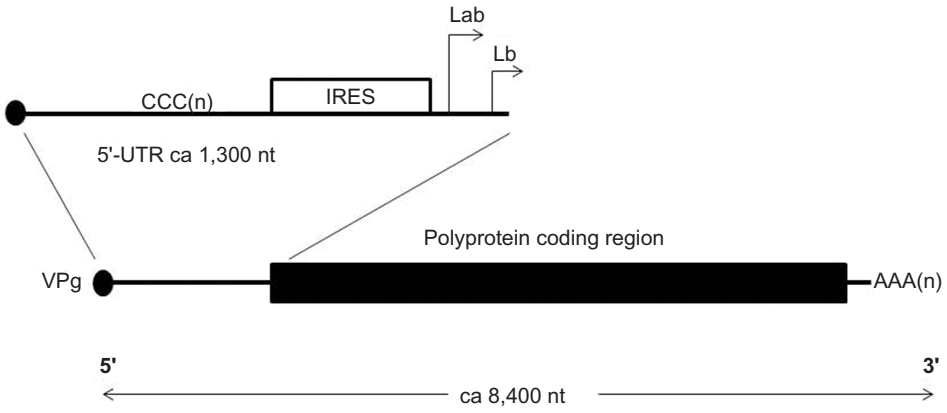

Figure 1 Structure of the FMDV genome.
Notes: The FMDV genome includes a single large open reading frame encoding a polyprotein. This coding region is flanked by the 5′-untranslated region (UTR) (ca 1,300 nt) and a 3′-UTR (ca 90 nt). The RNA includes a polyA tail at the 3′ terminus. The 5′ UTR includes a poly(C) tract, of unknown function, and the IRES which directs the initiation of protein synthesis on the viral RNA. Two initiation codons are used (separated by 84 nt) to produce two forms of the first part of the polyprotein (the leader [L] protein), termed Lab and Lb.
Abbreviations: FMDV, foot-and-mouth disease virus; RNA, ribonucleic acid; IRES, internal ribosome entry site; VPg, viral protein-genome linked; nt, nucleotides; ca, circa.

is enclosed within a roughly spherical protein shell (capsid) which is about 30 nm in diameter. The capsid serves to protect the genome while the virus is outside of cells and also allows it to bind and subsequently gain entry to cells through interaction with specific cell-surface receptors (eg, certain integrins). Interaction of antibodies against the surface of the virus capsid is considered to be the major mechanism for neutralizing FMDV infectivity. Thus vaccines to combat FMDV infection have been developed to generate antibodies against the capsid proteins. Current vaccines rely on the production of huge quantities of infectious FMDV, generally in large scale tissue culture systems, which is then chemically inactivated prior to inoculation into animals with an adjuvant.

The virus capsid is composed of 60 copies of four distinct proteins, termed VP1, VP2, VP3, and VP4 (see Figure 2), these are the structural proteins (SPs). The virus also produces eleven other different mature proteins (plus precursors) which are involved in the replication of the virus and/or combating host defense systems (see Characteristics of FMDV).

There are seven different serotypes of FMDV, namely O, A, C, Asia-1, Southern African Territories (SAT) 1, SAT 2, and SAT 3. There is no apparent cross protection between the serotypes, so animals that have been infected (or vaccinated) with one serotype of virus are not protected against other serotypes. Indeed, even within a single serotype there is considerable antigenic heterogeneity and thus vaccine matching studies have been undertaken to identify the efficacy of particular vaccine strains in generating antibodies that block infectivity of new outbreak strains.

The immunity conferred by vaccination against FMDV is quite short-lived;[2] it is commonly recommended, in areas with endemic disease, to revaccinate animals twice per year. In recent years, it has become usual to purify vaccine preparations to separate the virus particles (including the SPs) away from the non-structural proteins (NSPs). This can enable differentiation between animals that have been vaccinated (which will only have antibodies against the SPs) from those that have been infected (which can have antibodies against all the virus encoded proteins). However, vaccinated animals can still become sub-clinically infected which results in a low level immune response against the NSPs but a boost to the antibody response against the SPs.[3] Hence, this differentiation between infected and vaccinated animals (DIVA) is not perfect, especially since multiple rounds of vaccination, even with purified vaccines, can result in the induction of antibodies against NSPs which will be present at a low level in these vaccine preparations.

FMDV vaccine is heat labile and hence the maintenance of a "cold chain" for the vaccine before its administration is essential. A manufacturer may produce a good vaccine but if it is stored poorly then it may not function well when it is used and thus there is a need to determine vaccine quality, at the point of production, and also efficacy during vaccination campaigns.[4]

The text above indicates that there are a number of limitations and problems associated with current FMDV vaccines (eg, lack of cross protection, short duration of immunity, lability, requirement for high containment facilities to produce it) and thus there is interest in developing improved vaccines which address at least some of these shortcomings. It may well be that there will not be a single solution to these different issues and indeed the most important features of a vaccine

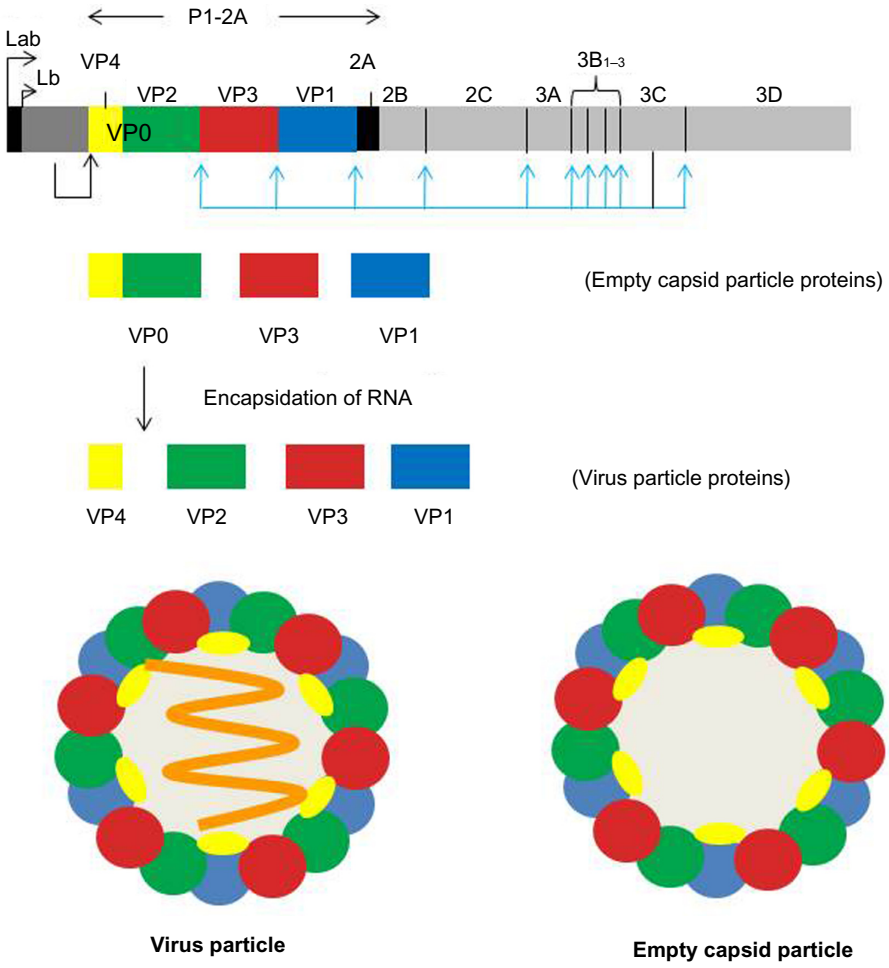

Figure 2 Schematic diagram showing the production and assembly of FMDV capsid proteins.
Notes: The virus encoded polyprotein is cleaved, mainly by the virus encoded 3C protease ($3C^{pro}$) but the Leader protein (L) cleaves the L/VP0 junction. The capsid protein precursor (P1-2A) is processed to VP0, VP3, VP1, and 2A (a short peptide) and these proteins can self-assemble to form empty capsid particles. During virus infection, production of mature virus particles includes the encapsidation of the viral RNA genome and during this process cleavage of VP0 to VP4 and VP2 occurs. Only VP1, VP2, and VP3 are exposed on the surface of the virus particle, VP4 is entirely internal. The RNA genome (indicated as an orange line) within complete virus particles is absent from empty capsid particles which are therefore non-infectious.
Abbreviations: FMDV, foot-and-mouth disease virus; RNA, ribonucleic acid.

may depend on their planned use. For example, in a country that is normally disease-free, the highest priority may be to use a vaccine which induces rapid protection against disease caused by the single type of virus that has been introduced. In contrast, in endemic areas (with poor infrastructure) which may have multiple serotypes of FMDV in circulation concurrently, it may be best to have a stable vaccine (without need for a cold-chain) which induces broad protection, ideally against multiple serotypes, if this is achievable.

Characteristics of FMD

FMD is highly contagious and affects cloven-hoofed farmed animals including cattle, pigs, sheep, and goats. It can also affect a large number (circa 70) of different wildlife species including, notably, the African buffalo (*Syncerus caffer*). Severity of clinical disease varies between the common domestic species, with pigs developing severe clinical illness, followed by cattle showing clear but less severe clinical signs, whilst the clinical course of the infection in sheep and goats may be very mild.[5]

The different serotypes of FMDV cause clinically indistinguishable signs of disease. The initial FMDV replication within infected animals is thought to occur within the epithelia of the pharyngeal mucosa.[6,7] From here, the virus spreads through the lymphatic and vascular system to peripheral sites of secondary replication, characterized by the presence of stratified, cornified, squamous epithelia, such as the coronary bands and oral cavity. Infected animals develop transient viremia lasting for 2–3 days, which is effectively counteracted by the development of circulating anti-FMDV antibodies. The clinical disease follows a rapid time course and typically includes a sudden rise in body temperature

and the development of vesicular lesions at peripheral areas of viral replication. Affected animals may display varying degrees of salivation, loss of appetite, and lameness according to the severity of lesions. The clinical course of the infection usually subsides within 7–14 days. However, many infected cattle (approximately 50%) may become FMDV "carriers"; these are defined as animals with asymptomatic, intermittent, presence of infectious virus in the oropharyngeal fluid for more than 28 days after infection.[8] Development of the "carrier" state is apparently unaffected by the presence of neutralizing antibodies in the circulation. Thus, both animals that are immunologically naïve at the time of exposure to FMDV, as well as those with circulating antibodies due to vaccination or previous exposure to the virus can become FMDV "carriers". The duration of the "carrier" state varies between species, with the longest duration recorded in African buffalo (5 years) followed by cattle (2 years) and sheep (9 months).[9,10] It is generally considered that pigs do not become "carriers"[5] but they may retain viral RNA for an extensive period (see below).[11] Since "carrier animals" are a potential source of infectious virus, their presence is considered unacceptable in areas free of FMD, however it has proven very difficult to demonstrate transmission of virus from carriers to naïve animals.[5,12]

Transmission of the virus can occur by a variety of different routes. Cattle can easily be infected by airborne virus whereas pigs need close contact with other infected animals since a gap of >70 cm is sufficient to block transmission of the virus from one pig to another.[13] In some instances, it appears that long distance (>100 km) transmission of the virus through the air to cattle has occurred; this has mainly been over water and may require specific weather conditions. During outbreaks, disease spread normally occurs on a relatively local basis (within a few km) but clearly transportation of infected animals can seed new outbreaks in diverse locations. Once infected, pigs exhale large quantities of virus and hence a common route of transmission is from infected pigs to cattle located downwind of these animals.[5]

Characteristics of FMDV

The FMDV life cycle is very short, indeed a single cell can be infected and produce $>10^5$ new virus particles in about 5 hours. The replication of the virus is carried out entirely within the cytoplasm of the cell and the viral RNA alone is sufficient to initiate an infection if it is delivered to the cytoplasm of cells.[14] The FMDV genome is about 8,400 nt long and encodes a large polyprotein from within a single open reading frame of about 7,000 nt (see Figure1). The uncapped viral RNA includes a very long (ca 1,300 nt) 5′-untranslated region (UTR) and a short 3′-UTR (ca 90 nt) plus a poly(A) tail.[15] The 5′-UTR contains a poly(C) tract, multiple pseudoknots (of unknown function), a cis-acting replication element (cre) (also referred to as a 3B-uridylylation site [bus])[16] and an internal ribosome entry site (IRES) which directs the initiation of protein synthesis on the viral RNA.[15] The full-length "polyprotein" is never observed since processing, largely by virus-encoded proteases, commences during the process of protein synthesis. In total 15 different mature polypeptides are made plus a variety of different precursor proteins some of which have functional significance with unique functions.

The initiation of protein synthesis on the FMDV RNA, directed by the IRES, occurs at two different sites separated, usually, by 84 nt.[15] This results in the production of two different forms of the Leader (L) protein, the first component of the polyprotein, and these are termed Lab and Lb. It has been shown that these two forms of the L protein share the known major functions of this papain-like cysteine protease.[17] They can both cleave the L/P1 junction, in trans and probably in cis as well. They can also both induce the cleavage of the eukaryotic initiation factor 4G (eIF4G) which is an essential component of the cap-binding complex. Cleavage of eIF4G results in separation of the N-terminal domain (that binds to the cap-binding protein eIF4E) away from the rest of the complex. The modified complex is still able to support cap-independent translation initiation on the FMDV IRES[15] and so FMDV protein synthesis can occur but does not support cap-dependent protein synthesis, hence translation of cellular messenger (m)RNAs is efficiently blocked.

The FMDV SP precursor, P1-2A, has a myristoylation signal sequence (GXXXS/T)[18] at its N-terminus and hence the N-terminal glycine residue (generated through the action of the L protease as indicated above) becomes modified by the addition of a myristate group through the action of the cellular modification system (see Figure 3). The presence of the myristate group appears important for the assembly/or stability of the capsid of FMDV and certain other picornaviruses.[19,20] The break at the junction between P1-2A and 2B is unusual; it requires the 2A peptide but this short, highly conserved, peptide lacks protease motifs and only functions to interrupt the formation of the polypeptide chain during polypeptide synthesis. It has been suggested that it induces "ribosomal skipping" at the NPG/P junction so that no peptide bond is actually made.[21] Processing of P1-2A precursor to produce VP0, VP3, VP1, and 2A is achieved by the 3C protease ($3C^{pro}$), and the final cleavage of VP0 to VP2 and VP4 occurs in the context of the assembled capsid. It used to

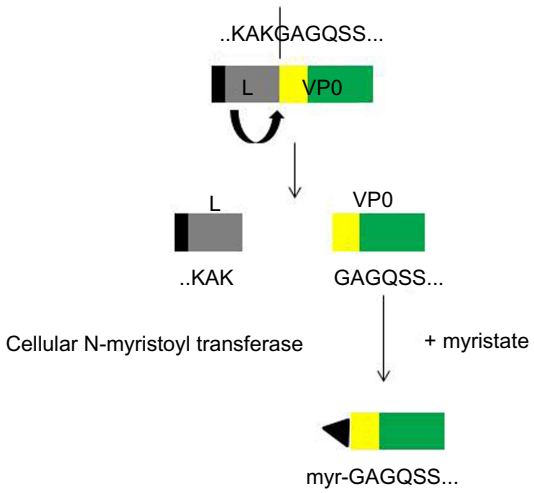

Figure 3 Myristoylation of FMDV capsid proteins by host cell systems.
Notes: Cleavage of the L/VP0 junction by the Leader (L) protease (indicated by arrow) generates an N-terminal glycine (G) residue on VP0 within the consensus motif (GXXXS/T) that is recognized by the cellular myristoylation system. Addition of the myristate (myr) moiety (indicated by ◀) is required for the formation of empty capsid particles and virions.
Abbreviation: FMDV, foot-and-mouth disease virus.

be thought that this cleavage was dependent on packaging of the genome, however, it has become apparent that it occurs within assembled "empty" capsid particles (lacking the RNA genome) too[22–24] but the mechanism is not known.

The P2 and P3 regions of the polyprotein include the NSPs. The functions of the P2 region (which is processed by $3C^{pro}$ to produce 2B and 2C) are not very well understood. It has been shown that 2BC or 2B together with 2C can inhibit trafficking of proteins to the cell surface,[25,26] this may have an adverse effect on the presentation of viral proteins to the host immune system, thus protecting the virus from immune defenses. The 2C protein is also implicated in viral RNA replication since it is the site of action of guanidine which can block this process. Mutants of the virus which become resistant to this inhibitor have mutations within the 2C coding region.[27–29] Recent studies have shown that the FMDV 2C protein is a member of the AAA+ family of proteins and forms a hexameric structure.[30]

The P3 region is processed to 3A, three different forms of 3B ($3B_1$, $3B_2$ and $3B_3$ which are often called VPgs [viral protein-genome linked]), 3C and 3D. The 3B peptides (23 or 24 amino acids each) are modified in an unusual reaction involving the covalent linkage of uridyl residues to a conserved tyrosine (Y) residue within each VPg.[31,32] The VPgpUpU then acts as a primer for the initiation of RNA synthesis and thus all picornavirus RNAs have a 5′-terminus comprising VPg-UU…. FMDV is unique in having three different versions of the VPg peptide and each is functional.[33]

The 3A protein may serve to deliver the VPg peptides to the RNA replication machinery.[32] Variants of FMDV with deletions within the 3A protein have been identified; these are associated with more restricted host range of the virus including attenuation in cattle.[34] As indicated above, the 3C protein is a chymotrypsin-like protease and is responsible for most of the proteolytic cleavages within the virus encoded polyprotein (see Figure 2). In contrast to poliovirus (PV) and other enteroviruses, which require 3CD as the active protease for P1 processing, the FMDV $3C^{pro}$ is sufficient for all processing events within P1-2A. The $3C^{pro}$ also has an RNA-binding region[32] and this may underlie its involvement (either alone or as part of the 3CD precursor) in the process of VPg uridylylation.[32] The 3D protein is the RNA dependent RNA polymerase ($3D^{pol}$) and hence is critical for the process of RNA replication; this involves the production of a negative strand RNA template and then many more copies of the positive strand genome.

Diagnostics for FMDV; current tests and detection methods

Suspected cases of FMD are identified based on clinical signs, including fever, excessive salivation, presence of vesicles on the oral mucosa, on the nose plus the inter-digital spaces and coronary bands on the feet. However, the clinical signs can be confused with other diseases (eg, vesicular stomatitis and swine vesicular disease) and thus a laboratory based diagnosis is required. The first priority is the specific detection of FMDV but it is also important to identify the serotype of virus involved in outbreaks (if vaccination is considered) and sequence analysis can be used to identify the potential source of the introduced virus.

Detection of FMDV RNA

The currently favored technique for the detection of FMDV is the use of reverse transcription quantitative polymerase chain reaction (RT-qPCR) assays; these are rapid, sensitive, and specific. Currently, two different RT-qPCR assays which recognize highly conserved parts of the genome are in common use, one targeting part of the IRES within the 5′ UTR[35] and the second targeting a region within the $3D^{pol}$ coding sequence.[36] These assays can be performed using robotic extraction of RNA and thus can achieve relatively high throughput, especially using one-step RT-qPCR procedures.[37] Thus the assays are suitable for the diagnosis of a primary index case and for use in an ongoing outbreak. However, these assays are not designed to discriminate between serotypes of FMDV and each of the assays can fail to detect particular

FMDVs due to the presence of nucleotide mismatches within the region targeted by the primers and probes. Thus, no single stand-alone assay is capable of detecting FMDV with 100% certainty. There has been recent work to develop RT-qPCR assays for serotyping of FMDV,[38] however due to the diversity of the viruses in different areas of the world it is likely that such assays will have to be tailored to specific global regions. Alternative nucleic acid amplification methods (eg, LAMP [loop-mediated isothermal amplification]) have also been described for the detection of FMDV RNA[39] which may be applicable in certain settings.

Virus isolation

Until the advent of RT-qPCR assays, the preferred technique for the detection of FMDV was the use of virus isolation followed by its characterization. Primary cell cultures (especially bovine thyroid cells) or certain cell lines (eg, BHK or IBRS2) are suitable for isolation of FMDV. Up to 4 days (using two passages of up to 2 days) may be required to demonstrate the presence of virus, especially when the initial levels of virus are low, and therefore it also takes 4 days to be confident that no virus is present.

FMDV antigen detection

Roeder and Le Blanc-Smith[40] established assays for the detection of FMDV antigen using high titer anti-FMDV antisera raised in rabbits and guinea pigs for antigen capture and detection, respectively. The assays are serotype specific and quick, thus they are frequently used for the diagnosis of FMD and for virus typing. However, the enzyme-linked immunosorbent assay (ELISA) is not very sensitive and only gives positive results with about 70%–80% of epithelial suspensions that contain the virus. Thus the virus may have to be propagated in cell culture (as described above) and subsequently tested in the ELISA to detect the virus and ascertain the serotype; clearly this significantly lengthens the procedure. Variants of this procedure also exist; for example, monoclonal antibody (Mab)-based ELISAs have been developed for detection and typing of FMDV[41] and a sandwich ELISA using recombinant integrin αvβ6 (a cellular receptor for FMDV) for virus capture and serotype-specific monoclonal antibodies as detecting reagents was reported.[42] These integrin/Mab ELISAs recognized FMDVs of broad antigenic diversity from all seven serotypes.

A Mab-based chromatographic strip test for FMDV detection was developed[43] as a pen-side test. This system can be as sensitive as the conventional antigen ELISA for the detection of FMDV in epithelial suspensions and may represent a convenient tool during outbreaks.

Serology

The virus neutralization test is considered as the "gold standard" for detection of antibodies to the SPs of FMDV and is a prescribed test for import/export certification of animals/animal products.[44] However, since primary cells and cell lines with variable degrees of sensitivities are used, these assays are prone to more variability than other serological tests. Furthermore, the virus neutralization test is slower and requires biocontainment facilities in contrast to other serological tests (ie, ELISAs) which use inactivated viruses as antigens.

Detection of antibodies against either the SPs or NSPs can be determined using ELISAs. As indicated above, vaccines consisting of purified preparations of chemically inactivated virus particles induce antibodies almost exclusively against the SPs of the virus (at least after a small number of vaccinations) but infection will induce antibodies against the NSPs as well. Thus it can be possible to discriminate between infected and vaccinated animals based on the detection of antibodies to NSPs. A species independent and serotype independent blocking ELISA using baculovirus expressed FMDV NSPs as antigen is commercially available and widely used. Serotype specific ELISAs for the detection of antibodies to the SPs are also described[44] and some are commercially available or as kits from reference laboratories.

It is clearly important that the design of new vaccines against FMDV should consider the importance of being able to differentiate between infected and vaccinated animals.

Current vaccines against FMDV and novel approaches that could elicit a better immunity

As indicated above, current vaccines rely on the production of huge quantities of infectious FMDV, generally in large scale tissue culture systems; the virus is then chemically inactivated and purified (optimally) prior to use, in combination with an adjuvant, as a vaccine. Properties of these vaccines have been reviewed previously.[2,45] Vaccination plays an important role in the control of FMD when outbreaks are frequent. For example, in Europe during the 1960s and 70s the disease was very successfully controlled using a combination of efficient veterinary services, movement controls, and vaccination. When the disease incidence became very low, vaccination was ceased and is no longer permitted in Europe except under emergency situations. It is clearly easiest to show the

absence of infection if there are no antibodies to FMDV within the animal population, however this does leave the animals fully susceptible if the virus is introduced. Certain countries in Europe (eg, the UK) have never vaccinated against FMD but the huge expense and loss of animals during the 2001 outbreak in the UK has led to consideration of new strategies.

Vaccination has been used in both the Netherlands and Japan in the face of outbreaks but in both cases all the vaccinated animals were then killed. "Vaccination to live", as an emergency strategy, has not been employed within Europe but it is foreseen that if new outbreaks occur in Europe there will be a high public/consumer demand for a non-killing strategy. In areas of the world where the disease is endemic, and no culling of infected animals occurs, vaccination is frequently used around reported outbreaks to reduce the spread of the disease. Shortcomings of the current FMD vaccine have been outlined above and thus there is significant interest in the development of improved vaccines against the disease which can assist in the reduction of the disease globally.

The most effective vaccines used to control other viral diseases (eg, against smallpox, rinderpest, and PV) have all been "live" attenuated viruses but it has not been possible to derive safe, effective infectious FMDV strains as vaccine candidates. This may partly reflect the diversity of the hosts for FMDV and the ability of the virus to change (and thus potentially revert to virulence). "Live" attenuated vaccines have the property of producing viral antigens within the infected cells and short peptides derived from such antigens are then displayed on the surface of infected cells in association with host MHC proteins, this is particularly important for the induction of cytotoxic T-cells. However, the role of cytotoxic T-cells in generating protection against FMDV is not clear[46] but induction of other T-cell responses may affect the duration of immunity.[47] Inactivated vaccines are recognized by antigen presenting cells and principally induce a B-cell response but this response is positively influenced by the presence of CD4(+) T-cells, since depletion of these cells reduces the level and nature of the antibody response.[48]

In the early days of genetic engineering it was very popular to try and express the FMDV VP1 in a variety of different forms, in numerous systems, to try and produce a non-infectious "subunit" vaccine. However, none of these proved very effective in inducing a good protective immune response in natural host animals. The unprocessed capsid precursor protein P1-2A does share some characteristics with the virus in terms of its antigenicity and it is able to bind to the integrin receptor ($\alpha v\beta 6$) that is used by the intact virus.[23,49,50] However, this precursor protein is not very immunogenic and fails to generate a protective immune response.[46,49]

Hence, more recently, the focus has been on expressing "empty capsid particles" (see Figure 2) since these non-infectious particles (lacking the viral genome) have the same antigenic and immunogenic properties as whole viruses[19,51] while having the advantage of not constituting a risk of spreading from the production plant. However, achieving the efficient expression of FMDV empty capsid particles has not proved completely straightforward (see below). In addition, unmodified empty capsids may suffer from some of the same issues as vaccines based on inactivated virus but should at least remove the need for virus growth under high containment facilities. The production of empty capsid particles does not need NSPs (other than $3C^{pro}$) and thus removes the possibility of these products inducing an anti-NSP immune response. This should improve the ability to discriminate infected from vaccinated animals. Furthermore, a good expression system may offer the possibility of modifying the FMDV capsid in a manner which is not possible when FMD virus viability is also required for the production system.

Primary requirements for FMDV "empty capsid" production

In principle, the production of FMDV "empty capsids" should require the co-expression of the P1-2A capsid precursor with the $3C^{pro}$, this can produce the processed products VP0, VP3, and VP1 which can self-assemble into particles (Figure 2). Indeed this was successfully demonstrated many years ago.[19] The system used at that time involved the use of two separate recombinant vaccinia viruses, one expressing the T7 RNA polymerase and another which contained the FMDV P1-2A+$3C^{pro}$ cDNA cassette downstream of a T7 promoter. When cells were co-infected with both recombinant viruses then production of FMDV "empty capsids" could be observed. However, this system is not suitable for use in animals as it is not possible to ensure co-infection of cells by the two separate viruses. Constitutive expression of the P1-2A+$3C^{pro}$ from a single recombinant vaccinia virus was not achieved since it was not possible to isolate such a recombinant virus; it appeared that the expression of this cassette had a negative effect on the growth of the virus vector.[52]

Secondary requirements for "empty capsid" production

Picornavirus RNA is produced and functions within the cytoplasm of cells, hence the nucleus is a "foreign" environment for it. The vaccinia virus expression system described above

is very suitable for the expression of picornavirus cDNA since all the transcription (to make RNA) occurs within the cytoplasm of the cell whereas the host cell transcription machinery is present within the nuclei and host cell RNA transcripts are extensively modified (eg, by splicing, capping, and polyadenylation) before the mature mRNAs are transported from the nucleus to the cytoplasm so that translation can occur. This means that picornavirus cDNA, and especially RNA transcripts derived from it, may be inappropriately recognized (eg, for splicing) within the nuclei of cells. It is worth noting that the initial system,[53] using cloned cDNA, to rescue PV used cDNA under the control of an SV40 promoter (which is only functional within the nucleus). The infectivity of this cDNA was very low, a few plaque forming units (pfu) were generated per μg of plasmid whereas systems that produce RNA transcripts from picornavirus cDNA in vitro can achieve $>10^5$ pfu/μg of RNA.[54] Thus, the transcription of picornavirus cDNA from within the nucleus appears to be very inefficient at achieving the cytoplasmic expression of intact picornavirus RNA and hence expression systems which avoid this may be preferable.

The amino terminus of the P1-2A capsid precursor is myristoylated (see above). This post-translational modification is achieved by a cellular system which is present within mammalian and insect cells but lacking in *Escherichia coli* (unless specific steps are taken to achieve this).[50] The assembly of the processed FMDV capsid proteins into pentamers (12S) and subsequently into empty capsid particles (75-80S) is inhibited if myristoylation is blocked;[19,50] similar observations have been reported previously for PV.[20,55,56] Thus it seems necessary to employ expression systems which permit this modification.

Successful expression of "empty capsid" components

As indicated above, the expression of P1-2A+3C^{pro} cassettes has been achieved in cells using vaccinia virus based vectors[19,51,52] and has been optimized by achieving reduced levels of the 3C^{pro} expression relative to the P1-2A.[23,24,51,57] These systems generate empty capsid particles which can be detected by sucrose gradient analysis and by electron microscopy. The structure of these particles has been determined by 3D-reconstructions from electron micrographs[23] and by X-ray crystallography.[51] Furthermore, despite some of the potential problems described above, it has been possible to express P1-2A+3C^{pro} cDNA cassettes using recombinant adenoviruses that direct transcription from within the nucleus.[58,59] No direct evidence for assembly of the expressed proteins into empty capsid particles has been reported using this system. However, it has been possible to achieve protection against FMDV challenge in animals using these vectors[60] but high levels of the adenovirus are required which may suggest that the expression of the FMDV proteins is relatively low, albeit detectable.[46] It is noteworthy that adenovirus vectors expressing serotype A (A24) cassettes have proved to be more successful than serotype O (O1 Campos) but the basis for this difference is not defined.[61]

The baculovirus expression system also uses transcription from within the nucleus but most baculovirus transcripts are not spliced and it therefore appears that the potential for adverse modification of transcripts derived from FMDV cDNA within recombinant baculoviruses is more limited.[62] However the baculovirus expression system has proved sensitive to the activity of the FMDV 3C^{pro}. Thus it has required introduction of both a frameshift signal (to reduce 3C^{pro} expression level) and introduction of an amino acid substitution, to reduce protease activity,[63] to allow satisfactory expression and processing of the P1-2A precursor into empty capsids.[51,64] This system has permitted the expression of a stabilized form of FMDV empty capsids (with a single amino acid change in VP2) which display tolerance to heat or low pH treatment and are able to induce protection against FMDV challenge.[51] A feature of the baculovirus system is that the virus can be grown in insect larvae and generate large amounts of protein.[65–67]

A recent report has described the co-expression of VP0, VP3, and VP1 as His-tagged SUMO (small ubiquitin-like modifier) fusion proteins in *E. coli*.[68] After removal of the His-SUMO moieties using SUMO protease, apparent assembly of the capsid proteins into virus-like particles (ca 25 nm diameter), that sedimented at 75S, was observed. These were able to induce protection in guinea pigs, pigs, and cattle against FMDV challenge. Precise details of the protein sequences present within these particles are lacking and the issue of VP0 myristoylation was not addressed. It is therefore unclear how these particles have assembled.

The role of NSPs, as well as other elements, for the development of more effective vaccines

Recent studies[61,69] have demonstrated that inclusion of the FMDV 2B coding sequence into the P1-2A+3C cassettes expressed from recombinant adenovirus vectors have improved the properties of both serotype A and O cDNA cassettes. The analyses indicated that improved T-cell responses were obtained but there was no difference in the level of neutralizing antibodies generated. A rather different strategy for

enhancing the immune response to FMDV vaccines has been described[70] in which RNA transcripts, including the IRES, were delivered in conjunction with commercial inactivated FMDV vaccine and found to enhance the anti-FMDV antibody titers in mice. It was suggested that the RNA transcripts acted like an adjuvant. The RNA transcripts were found to be potent type-I interferon inducers and this may underlie this effect. It is well known that interferon has a strong inhibitory effect on FMDV replication and studies have shown that expression of interferon, from adenovirus vectors, can effectively block FMDV replication in swine.[71]

The precise deletion of the Lb coding region to make a so-called "leaderless virus" (see Figure 4) from FMDV results in a virus which is still viable[72–74] but attenuated in cattle.[75] The loss of Lb results in a decreased ability of the virus to shut off host cell protein synthesis; this will allow the synthesis of interferon.[76] Such viruses can grow well in BHK cells[73,74] and may represent useful tools for the development of safer vaccine strains for conventional cell culture production since any escape only releases viruses that are non-pathogenic and cannot revert to virulence.

There has been significant interest recently in generating marked FMDVs which may permit improved DIVA and/or offer alternative routes for virus purification. Sequences for the HA and FLAG epitope tags were inserted into the VP1 within the virus capsid without adverse effect on virus growth.[77] In an alternative strategy, it has been shown that the cleavage of the VP1/2A junction (by $3C^{pro}$) is not required for virus viability[24,78] and the modification of the cleavage site can result in the formation of "self-tagged" virus particles which contain the VP1-2A fusion protein. Thus no "foreign" sequences are added to the genome but the presence of the 2A attached to the VP1 can be readily detected (using anti-2A antibodies). The epitope-tagged viruses and "self-tagged" viruses offer the possibility of alternative (serotype independent) purification systems for virus antigen. They should also induce a distinct immune response compared to the native virus which could be used as part of a DIVA system. However, use of such "tags" does not allow the identification of animals which have been vaccinated and then infected. It is important to note that current FMDV vaccines do not prevent infection but only disease; indeed vaccinated animals can become "carriers" (see above).

Alterations in the immune response that are caused by infection with FMDV

The major focus of the immune response against FMDV is production of antibodies that can recognize and neutralize the virus. Such antibodies are generated within 4–7 days of infection and their detection within serum is coincident with the cessation of viremia within infected animals. Antibodies to NSPs are also generated but these tend to appear a little later.[79] The role of T-cell responses in immunity against FMDV is rather poorly understood. Infection of cattle does not appear to have an adverse effect on the ability of these animals to mount an immune response against FMDV,[80] however a transient loss of circulating T-cells has been reported in swine.[81]

As mentioned above, in about 50% of infected cattle, after the acute phase of infection which normally resolves within about 14 days, a long-term "carrier" state can occur for up to 2–3 years. It occurs despite the presence of neutralizing antibodies in the serum. Since FMDV does not

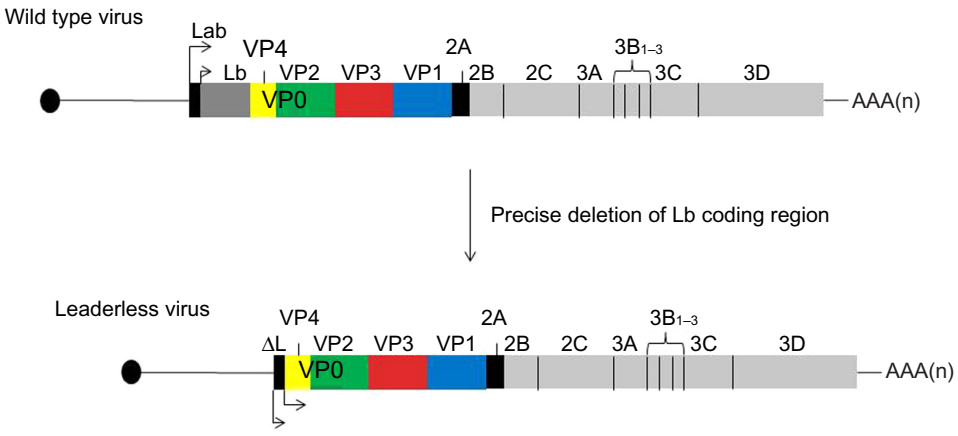

Figure 4 Production of attenuated "Leaderless" FMD viruses.
Notes: Precise deletion of the Lb coding region from the FMDV polyprotein coding region generates a "Leaderless" form of the virus which has been shown to be attenuated in cattle. Such viruses can still replicate efficiently in cell culture and may allow the safer production of FMDV vaccines.
Abbreviations: FMDV, foot-and-mouth disease virus; FMD, foot-and-mouth disease.

survive for very long at 37°C,[82] it seems that the virus must be replicating within the carrier animal in a site which is protected from the host immune response. The site of such replication is not precisely established; both the dorsal soft palate and germinal centers of lymph nodes have been identified as sites of long-term virus maintenance[83,84] but biopsy samples taken from both of these sites from "carrier" cattle that were excreting virus failed to detect the viral RNA in most of these tissue samples.[85] It seems unlikely that cells can tolerate a long-term infection by FMDV, the loss of host cell protein synthesis which occurs at very low levels of FMDV L protein expression, should be lethal. This either suggests that infection of fresh cells is occurring on a regular basis, albeit probably involving a small number of cells at any one time, or that a mutant form of the L protein is expressed by the viruses within carrier animals but there is no evidence for this.

The germinal centers of lymph nodes from cattle and buffalo that have been infected by FMDV can contain viral RNA and protein for extended periods of time but this is believed to be in a non-replicating form[84,86] and it is not known whether such virus can infect other cells. As indicated above, although it is generally believed that pigs do not become carriers, evidence has been presented that the FMDV RNA and SPs can also be detected in porcine lymph nodes for some weeks after infection[87] but no infectious virus could be recovered from these tisssues.[11,88]

Immune response elements that might contribute to protection against acute and persistent infections

FMDV replication is highly sensitive to type I interferon and, as described above, the virus has a mechanism for stopping virus-infected cells from producing interferon (and other host proteins) by blocking host cell protein synthesis. However, within FMDV infected cattle it has been possible to detect interferon within the serum of acutely infected cattle.[89,90] It therefore seemed possible that the interferon detected did not originate from FMDV infected cells but potentially from other cells that detected some of the viral components. It has been reported that bovine plasmacytoid dendritic cells are the major source of type I interferon in response to FMDV infection.[90] However, these cells are believed to interact with viruses within immune-complexes and it is not clear whether these would be present during the acute phase of infection when the interferon response is observed,[89] some days before anti-FMDV antibodies are detectable in serum.

One process that may contribute towards the ability of FMDV-infected cells to be maintained within the host is the ability of the 2BC protein (or 2B and 2C together) to block the transport of proteins to the cell surface.[25,26] This can have the effect of blocking virus derived peptides from being transported and then displayed on the cell surface and hence will render the infected cell apparently "normal" and hence not subject to immune surveillance.

Changes in cellular signaling pathways triggered by FMDV

When FMDV infects cells it can initiate a variety of intracellular responses. These could be triggered by interaction of the virus with the cell-surface exposed integrin receptor, through the introduction into cells of highly structured RNA, through the shut-off of host cell protein synthesis (which can induce apoptosis), and by direct effects of viral proteins on host cell systems. The virus can combat such host cell responses directly, eg, by inducing cleavage of specific proteins involved in such events, or more indirectly (but in a general fashion) by blocking host cell macromolecular biosynthesis (transcription and translation). Some effects of virus infection appear to be a combination of such processes. Interferon induction can be blocked by inhibiting host cell transcription and translation but there are also reports that the FMDV L protease induces cleavage of NF-κB[91] while the $3C^{pro}$ cleaves the NF-κB essential modulator (NEMO), a bridging adaptor protein required for the activation of both NF-κB and interferon-regulatory factor signaling pathways.[92]

Conclusion

Current vaccines developed to control FMD have significant shortcomings. However, when used appropriately and in conjunction with other control measures, they can be very helpful in controlling and even eradicating the disease on a regional basis (as achieved in Europe). However, it is likely that improved FMDV vaccines will be required to achieve the global eradication of the disease. Different situations may require different solutions but it seems likely that the production of non-infectious, specifically engineered, "empty capsid particles" using a suitable expression system will form the basis of such improved vaccines.

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Disclosure

The authors declare no conflict of interest.

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