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Review Article

Problems in FMD Eradication: A Way Forward?

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Abstract

Foot and Mouth Disease Virus (FMDV) is one of the most commonlyreported OIE-listed pathogens. FMDV is one of the most contagious mammalian viruses known to man: the virus is endemic in many developing countries causing substantial economic loses and the restriction of international trade in animals/animal products. The virus infects domestic animals (cattle, pigs, sheep, goats), but also a wide range of wild-life species, the latter forming reservoirs of disease. The need to diagnose FMDV infections (serotype/strain identification) and vaccine production/testing requires expensive, high disease security/containment facilities. Chemically inactivated ('killed') vaccines have been available for decades, but the huge genetic diversity of this virus (7 serotypes with 1000s of subtypes) and the need to periodically re-vaccinate animals to maintain protective levels of antibodies argue for the development of new vaccines. Indeed, in the early 1990s, on the basis of a cost-benefit analysis, the European Union replaced the policy of routine vaccination using the inactivated vaccine with disease control via mass-slaughter of infected and surrounding susceptible animals (plus vaccination in extremis). For various reasons mass-slaughter is unacceptable in many developing countries so in their case vaccination, in one form or another, is the only way forward. In the past few years there have been exciting developments in the production of new types of vaccine and many hold great prospects for improving disease control, but the thesis of this paper is that only the development of a new type of vaccine – live, attenuated, FMDV strains, offers the prospect of eradicating FMDV.

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- Virus-like particles
- Live, Attenuated FMDV vaccines

ABBREVIATIONS

BEI: Binary Ethyleneimine; BHK: Baby Hamster Kidney Cells; cDNA: Complementary DNA; FMD: Foot and Mouth Disease; FMDV: Foot and Mouth Disease Virus; GMO: Genetically Modified Organism; OIE: World Organization for Animal Health, Office International Des Epizooties; Vlps: Virus like Particles; vRNA: Virus Ribonucleic Acid; SAVE: Synthetic Attenuated Virus Engineering; ZAP: Zinc-Finger Antiviral Protein

INTRODUCTION

Foot and Mouth Disease (FMD) is one of the most devastating animal diseases, affecting agricultural productivity across the globe. This disease is causedby one of the most contagious viruses known to man, Foot-and-Mouth Disease Virus (FMDV). Year upon year, FMD is one of the most frequently notified OIE listed diseases. The virus infects a wide range of animals domesticated and over 70 wild-life species. Although mortality is usually low in adult animals, following resolution of the acute phase of the infection, long-term sequelae reduces animal productivity (e.g. milk yield) and, in developing countries, other agricultural activities through a reduction of draft-power. FMDV (family *Picornaviride*; genus *Aphthoviridae*) comprises seven, immunologically distinct, serotypes; A, O, C, Asia1, SAT 1, SAT 2 and SAT 3 although type C has not been isolated in the wild for over a decade. Each of these serotypes comprise numerous subtypes: although subtypeswithin a serotype are immunologically cross-reactive to some degree, infection by one subtype may provide partial, or essentially no, protection against infection by another subtype. This level of antigenic variation means controlling the disease by vaccination is highly problematic since multiple serotypes exist and variation within a serotype can render available vaccines ineffective. Indeed, this was amply demonstrated by the extensive outbreaks of FMD within several countries in Western Europe during 1965-1966 when a serotype O virus 'broke-through' the immunity of cattle vaccinated previously in comprehensive control programmes.

A factor which has played a rolein developmentof both disease control strategies and restrictions on international trade in animals / animal productsis that,following the acute stage of infection, in a proportion of animals (naive or vaccinated) a persistent infectionmay be established – the 'carrier-state'. It has long been thought that such animals may act as foci of new outbreaks. Whilst transmission from carrier African Buffalo to cattle has been demonstrated, experiments on virus transmission from carrier domestic livestock to companion naive animalsprovided no such evidence (reviewed in [1-4]). The establishment and duration of the carrier state has been shown to be related to virus virulence [5], although changes in the host-cells may also play an important role [6].

In contrast to another picornavirus, poliovirus, it can be seen that FMDV disease control let alone eradication is made a very much more complex taskby the huge genetic diversity, the existence of wild-life virus reservoirs and the carrier state.

Disease control strategies

The freedom to trade in animals / animal products is governed by the status of a country, or, a zone within a country: this may have a major impact upon the agro-economy of a country/zone. These different status comprise; FMD free countries/zones where vaccination is not practiced (no restrictions placed upon trade), FMD-free countries/zones where vaccination is practiced (trade restricted) and an FMD-infected country/zone (trade restricted). In the first case animals do not possess any anti-FMDV antibodies, whilst in the second case vaccination produces anti-FMDV antibodies - raising the possibility that these may have arisen from an undetected infection and, therefore, animals / animal products may harbor virus. The decision to control FMDV by routine vaccination has, therefore, a substantial effect upon international trade.

Mass-Slaughter or 'De-Population' of infected/ susceptible animals: Here, animals on infected premises and susceptible animals identified as 'dangerous contacts" (e.g. contiguous premises, or, animals within a certain radius), are culled humanely and the carcasses incinerated, rendered, or disposed of within licensed commercial landfill sites. In the 2001 UK outbreak, for example, some 6,134,078 animals (cattle, sheep, pigs, goats, deer etc.) were slaughtered .Vaccination would, however, be considered as an extra control measure if it were thought this would help to control and eradicate the disease. These measures are accompanied by livestock movement bans, restricting public access, comprehensive cleansing and disinfection and heightened biosecurity measures at the national level.

In many developed countries FMDV has been eradicated from domestic animals and, importantly, any potential wild animal reservoirs of disease. Here, mass slaughter (see below) to eliminate FMDV results in regaining the OIE 'FMD free country where vaccination is not practiced' (and hence freedom to trade) more rapidly than would be the case if vaccination was used to control an outbreak: on average, the OIE recognizes FMD-free status ~6 months (200 days) after the last reported case of FMD. In the case of the 2001 UK outbreak (Feb 20th to Sept 30th) it took 114 days for OIE Scientific Commission to confer 'disease freedom' recognition, and in the case of the 2007 UK outbreak (July 29th to Sept 30th), again it took 114 days. However, in many developing countries the policy of control *via* mass-slaughter is not feasible upon economic, sociological, ethical or religious grounds. On a purely pragmatic level, the existence of (multiple) wild-life animal reservoirs of FMDV in many developing countries makes mass-slaughter unfeasible as a control strategy essentially locking them into vaccination in one form or another and, therefore, the concomitant restrictions on international trade.

Chemically inactivated vaccines: In the early-mid 1960s, the adaptation of baby hamster kidney (BHK) monolayer cells to growth in suspension culture within large-scale fermenters (x1000s litres), combined with advances in purification protocols, allowed the industrial-scale production of FMDV particles. Contaminating cellular proteins/ cellular debris, released during cell lysis, are removed by filtration and industrial-scale chromatography to purify the virus particle antigens. Purified particles are then chemically inactivated, usually using binary Ethyleneimine (BEI). To achieve satisfactory vaccine potency, these inactivated virus preparations must be formulated with adjuvants: aluminium hydroxide supplemented with a second adjuvant, saponin, are routinely used in aqueous vaccines for ruminants whereas oil emulsion vaccines are widely used for the immunisation of pigs which respond poorly to the aqueous vaccines. To maintain protective levels of antibody, commonly animals receive a booster vaccine within ~1 monthof the first vaccination followed by subsequent boosters every 4-6 months or less frequently depending on the prevalence of the disease in the region. The need for repeated administration of the chemically inactivated vaccine increases the cost an important factor in developing countries, plus the use of chemically inactivated vaccines does not address the problem of wild-life reservoirs of disease. Another key point here is thecloseness ofthe 'match' between the strain of virus used to produce the vaccine and the strain of the infecting virus – quite simply, the more similarity between the antigenic structures of the two viruses, the better the protection. The presence of viruses of multiple different serotypes or subtypes in regions comprising developing countries adds to the cost and reduces the potential efficacy of FMDV vaccines.

Subunit vaccines / virus-like particles: Antigenic sites on the virus particle were mapped using a combination of a range of techniques. For example, sera from infected animals were probed for binding to an overlapping panel of synthetic peptides ('pepscan'), sequencing anti-FMDV monoclonal antibody 'escape' mutants, and identification of regions of hyper-variation by sequencing naturally-occurring variants and by the resolution of the atomic structure of the virus. Much research was conducted into subunit vaccines basedupon (i) synthetic peptides (alone or conjugated to various carriers) corresponding to the major immunogenic sites on the virus particle, or, such sequences genetically fused onto other virus particles, and (ii) individual capsid proteins expressed in a rangeof heterologous expression systems for use directly as immunogens, or, in attempts to assemble virus-like particles (VLPs) *in vitro*. To summarize these experimental approaches, peptides or individual proteins are insufficiently immunogenic to be used as vaccines.

Experience has shown that virus particles or virus-like particles (VLPs) are by far the best immunogens. FMDV encodes its proteins in the form of a polyprotein which is proteolytically 'processed' by a virus encoded proteinase, 3C^{pro}. Virus particles consists of 60 copies each of four structural proteins (VP1–VP4) which are formed by processing of a precursor form ([P1-2A]) by 3C^{pro}. Five copies self-associate to form a pentamer, 12 copies of which subsequently self-associate to form the complete particle. To assemble a VLP, therefore, one needs to co-express both the [P1-2A] and 3C^{pro}. However, FMDV 3C^{pro} is highly cytotoxic and direct genetic fusions to co-express the substrate ([P1-2A]) and the 3C proteinase gave poor yields of VLPs in heterologous expression systems due to this cytotoxicity. A further approach was to use a replication-defective human adenovirus type 5 (Ad5)

vector encoding the FMDV P1-2A capsid coding region linked, by other FMDV sequences, to FMDV $3C^{pro}$. A single administration of such vectors produced immunity to virus challenge, although high doses of these vectors are neededwhich may limit their use in the livestock industry [7-10]. Here, the animal is essentially acting as a 'bioreactor' to generate its own vaccine. With regards heterologous expression systems, an elegant solution to FMDV $3C^{pro}$ cytotoxicity was developed by introducing a ribosomal frame-shifting site between sequences encoding [P1-2A] and $3C^{pro}$, together with a site-directed mutation within the active site of $3C^{pro}$ to reduce its activity. This strategy produced high-level expression of the [P1-2A] precursor together with reduced expression of the $3C^{pro}$ (with reduced activity). In this manner, the cytotoxicity of $3C^{pro}$ was reduced, but with sufficient activity to process [P1-2A]: the yield of VLPs was greatly enhanced [11]. This approach was further refined to address the acid and thermo stability of the VLPs. FMDV particles dissociate into pentamers at pH<7.0 and elevated temperatures: reducing the shelf life and necessitating a cold-chain for vaccine distribution. Knowledge gained from the atomic structure of the virus was used to introduce a site-directed mutation such that an amino-acid substitution produced much more stable capsids by the formation of disulphide bonds across the inter-pentameric interface of the particle. These capsids are now thermostable ($56^{o}C$) and acid stable (down to pH 5.2). Critically, these VLPs proved to be highly immunogenic and protected cattle against challenge with wild-type virus [12]. This approach has a number of merits; (i) it is completely bio-secure –infectious FMDV is not involved at any stage in the production process: VLPs can be produced out with high-containment facilities, (ii) the system can be adapted rapidly to express VLPs for any FMDV strain once the sequence encoding the capsid proteins has been determined, (iii) commercially viable levels of VLP expression can be achieved using insect cells (although cost-per-dose could well be an issue here, particularly for developing countries)and (iv) these modified VLPs can be stored and distributed much more easily. However, the strategic problem of virus within wild-life animal reservoirs remains.

Live, attenuated, vaccines: To date, two viruses have been globally eradicated Smallpox and the animal virus Rinderpest. Both were eradicated using live, attenuated, vaccines. In the case of Rinderpest this involved eradication of the disease in wild life reservoirs. In the 1920s Rinderpest was modified by serial passage in goats. After 600 such passages the virus was sufficiently attenuated such that vaccination with this virus eliminated the morbidity in healthy cattle (but not completely within immuno-compromised animals) and conferred life-long immunity. This live, attenuated, vaccine also had the advantage that it could be freeze dried for storage and distribution. The huge success of the live, attenuated, Poliovirus vaccines developed by Albert Sabin in the 1950s inspired new experiments to develop such vaccines for animals. This'classical' approach of attenuation by serial passage in tissue-cultured cells, so successful for Poliovirus, was adopted for Rinderpest and FMDV. In the case of Rinderpest, by the 90^{th}serial passage in calf kidney cells a virus was produced that did not cause disease, but did confer protective immunity – the Plowright vaccine. The attenuated virus was genetically stable and did not back-mutate to a virulent form. Not until much laterthe nucleotide sequence of the genomes of the original, highly virulent, Kabete 'O' strain and the Plowright vaccine strain was determined and, using molecular biology, a panel of recombinant (Kabete/Plowright) virus genomes was constructed to map the attenuating mutations. Viruses were rescued from this panel of infectious copies ('reverse genetics') and the virulence of each of these 'chimeric' viruses determined. It was concluded that attenuation was based upon the accumulation of mutations that had occurred within most of the virus genes – hence the genetic stability [13]. This live, attenuated, vaccine was responsible for the global eradication of Rinderpest.

In the 1960s a similar approach was adopted for FMDV: serial passage of FMDV in tissue cultured cells plus evaluation of attenuation using a mouse model system. These candidate 'attenuated' viruses were tested in cattle in Africa, but were found to cause disease [14-21]. The accumulation of mutations during the serial passage of virus in tissue cultured cells is a stochastic process andthe attenuatedphenotype may arise from only a small number of 'key' mutations within the genome. Upon further virus replication of the candidate vaccine strain in the production/amplification process or in the recipient back-mutation of these few key mutations can result in the virus regaining virulence. Alternatively, attenuation in the model system may not reflect attenuation in the target species. Whatever the cause, these failures brought about the notion that one could not produce live, attenuated, strains of FMDV.

Modern molecular biological techniques, including synthetic biology, plus the development of FMDV infectious copies allows us to manipulate the (vRNA) genome of FMDV at the level of a genome-length cDNA copy and to 'rescue' virus from these modified genomes. However, can one 'design' a genetically stable attenuated genome? FMDV has been attenuated by deletion of the L proteinases (L^{pro}), mutation of the SAF-A/B, acinus, and PIAS (SAP) domain within $L^{pro}$and by transposition or deletion of vRNA secondary structures [22-26]. In the case of the L^{pro} SAP-domain mutant virus inoculation of pigs did not cause clinical signs of disease, viremia, or virus shedding even when inoculated at doses 100-fold higher than that required to cause disease with wild type virus. Furthermore, SAP domain mutant virus inoculated animals developed a strong neutralizing antibody response and were completely protected against challengewith wild-type FMDV as early as 2 days post-inoculation and for at least 21 days post-inoculation. It should be noted, however, that in this case attenuation is based upon only 2 mutations ($Ileu^{55}$→Ala plus Leu^{58}→Ala) - although these mutations were stable over 5 passages in tissue-culture. The problem associated with the approaches outlined above is that they are 'inflexible', in that only a one, specific; level of attenuation can be achieved by any such individual genome manipulation. In contrast, the method of attenuation described belowallows one to finely controlthe level of attenuation for each target species.

A powerful new approach Synthetic Attenuated Virus Engineering (SAVE) allows us to control the level of attenuation. Here, 'synthetic biology' (the ability to chemically synthesis 1000s of bases in asingle gene block) enables us to redesign the FMDVgenome by introducing literally hundreds of synonymous (silent) mutations. The degeneracy of the genetic code enables

genome modification such that no amino acid is changed within any virus protein, but the nucleotide sequence encoding these proteins can be radically altered. For example, the genome of poliovirus was modified to substantially alter the codon-pair bias. Two viruses were created (PV-Min XY and PV-Min Z) with 407 and 224 synonymous mutations, respectively. Upon repeated passage of these attenuated viruses in tissue-culture HeLa R19 cells, no change in the phenotype was detected, nor nucleotide changes observed in the 'synthetic' region of the genome [27]. Attenuation arises from hundreds of mutations– each of which only very slightlyreduces the ability of the virus to replicate withincells but, taken together, produce a significantlevel of attenuation.This approach overcomes the problem of backmutationto virulence (a few back-mutationswould have no substantive effect).However, it is known that in RNA viruses, such as FMDV and polio, there is a marked suppression of the frequencies of –CpG- and –UpA- dinucleotides [28-31]. At variance with the notion that altering the codon pair bias is basis of the 'attenuating principal', it was shown that increasing the frequency of the dinucleotides –CpG- and –UpA- within the genome (and not codon pair bias) was the basis of attenuation [32]. In support of this interpretation, it was shown that the cellular protein zinc-finger antiviral protein (ZAP) inhibited HIV-1 virion production by cells infected with –CpG- enriched HIV-1. Furthermore, HIV-1 mutants containing segments whose –CpG-dinucleotide frequency mimicked a random nucleotide sequence were defective in un-manipulated cells, but replicated normally in ZAP-deficient cells [33]. Viruses appear to evade this cellular innate immune response by suppressing these dinucleotide frequencies. In answer to the question posed above "can one 'design' a genetically stable attenuated genome?". The answer certainly appears to be 'yes'! Like the technologies underpinning the production of VLPs, modern molecular biological techniques would allow the rapid creation of an attenuated vaccine which corresponded to any FMDV strain again, once the sequence encoding the capsid proteins has been determined.

We, and many others, have advocated this approach to generatelive, attenuated, vaccines against a range of viruses.In studies performed on FMDV at the Plum Island Animal Disease Centre, the region of the FMDV A12 genome encoding the capsid proteins (P1) was modified such that 489 synonymous mutations were introduced. The virus rescued (A12-P1 depot) was genetically stable and in tissue cultured cells grew to a similar titre as wild-type virus, but with a reduced specific infectivity. Interestingly, studies in mice showed that all of these animals survived inoculation with A12-P1 depot at 100 fold dose higher than that of a wild type virus causing 100% lethality. Furthermore, all animals inoculated with A12-P1 depot mounted a strong antibody response and were protected against a subsequent challenge with a lethal dose of wild type virus (21 days post-inoculation). Similar data were produced for pigs: clinical signs in animals inoculated with A12-P1 depot were not observed using 10^3 to 10^4 fold dose higher than that of wild type virus which caused severe disease within 2 days [34]. This work undoubtedly shows the huge potential of such a live, attenuated vaccine.

Introducing the mutations within the P1 region means, however, that the process of introducing such mutations would need to be repeated for each (new) strain, probably with subsequent characterisation. We have adopted the strategy of introducing such mutations not within P1, but into the region of the genome encoding the replication proteins (P2 and P3) to create an attenuated, pre-characterised, 'backbone' into which any P1 sequence could be inserted cutting down the response time in the case of novel outbreak strains.

The key questions that remain to be answered include;

(i) Can we achieve the correct balance between a reduction (hopefully elimination) of the morbidity of such live, attenuated, vaccine strains whilst retaining replication within the animal sufficient to produce a protective immune response? Experience with other live, attenuated, viruses (e.g. Rinderpest, Poliovirus, Yellow Fever Virus, Measles, Mumps, Rubella) supports the notion that indeed one can achieve such an outcome. In the case of vaccines produced by SAVE, the degree of attenuation would be 'regulated' through manipulation of dinucleotide frequencies*via* reverse genetics and synthetic biology.

(ii) Importantly, would this balance be the same for all target species (pigs, cattle, sheep, and goats)? The wide host-range of FMDV is a potential complicating factor: however, Rinderpest also had a wide host-range (domestic cattle/pigs, wildebeest, waterbuck, warthog, eland, kudu, giraffe, deer, various species of antelope, hippopotami, and African buffalo) but the Plowright attenuated vaccine eradicated the virus in all these animal reservoirs.

(iii) Would this type of vaccine be genetically stable? The useof SAVE to produce the next generation of live, attenuated, vaccines produces very high genetic stability compared to such vaccines produced by the 'classical' method.Given that SAVE attenuation is thesummation of literally hundreds of mutations, back mutation of a modest number of nucleotides will not change the attenuated phenotype.Specifically with regards FMDV, the experience of the Plum Island studies indicate SAVE vaccines would be genetically stable.

(iv) Could this type of vaccine generate life-long immunity? The great advantage of live, attenuated, vaccines over alternatives is that the recipient mounts an immune response (both B- and T-cell) against both structural *and* non-structural proteins. Naturally, this presents a problem for differentiation between vaccinated *versus* infected animals but we, and others, are working on the inclusion of novel epitopes into new vaccines to enable the differentiation between vaccinated and infected animals (DIVA). The duration of protection is a known short-coming of chemically inactivated vaccines the duration of protection using live, attenuated, FMDV vaccines is unknown, but experience with such vaccines against other viruses is encouraging.

(v) Could vaccinated animals transmit the attenuated virus to naive companion animals (or wild-life species): is this a bad or a good thing? In the case of the Sabin'classical' poliovirus attenuated vaccine it was suspected for many years that, in comparison to the vaccine strains (Sabin strains 1-3), there were adverse changes in the phenotype of virus excreted by vaccines. Not until genome sequencing technologies were developed was it determined that, indeed, antigenic changes in type 1 and an increase in the virulence of the type 3 strain did occur: poliovirus

excreted from vaccines could cause disease a bad thing! [35-37]. However, unlike the live poliovirus vaccines where a modest number of mutations confer the attenuated phenotype, this would not be the case for FMDV vaccines where the phenotype would be based upon the cumulative effect of literally hundreds of mutations: any potential transmissionof such genetically stable vaccine strain between animals (domestic or wild-life) would only produce an increase in the sero-conversion rate a good thing!

(vi) Would recombination between live, attenuated, vaccine strains and circulating virulent viruses either exacerbate or ameliorate the problem or neither? In countries where FMDV is endemic, the use of live, attenuated, vaccines obviously would entail possible recombination between the circulating wild-type and the vaccine strain: the advantage of the modern molecular techniques used in SAVE is the ability to rapidly produce an exact 'match' between the vaccine strain capsid protein sequences and those of the circulating wild-type virus. Progeny viruses from any such recombination event would not produce a more pathogenic virus.

(vii) Could the administration of a live, attenuated, vaccine lead to the establishment of the carrier-state? It is paradoxical that one of the most rapidly replicating, lytic, mammalian viruses is able to establish persistent infections. Vaccination with a live, attenuated, vaccine would produce a full range of cytotoxic T-cell responses (unlike chemically inactivated vaccines) that would eliminate the attenuated virus and prevent the establishment of persistent infections.

(viii) Would attenuating mutations within such vaccines introduced using molecular biological techniques be substantively different than mutations accumulated by serial passage in tissue culture cells: would the SAVE vaccines be classified as genetically-modified organisms (GMOs)? A distinction without a difference? This is, essentially, not a scientific but a political question. SAVE vaccines are designed rationally whilst 'classical' attenuated vaccines are produced by a stochastic process. The debate over GMOs continues: whilst many EU countries oppose growing genetically modified (GM) crops, the EU imports millions of tons of GM crops per year for animal feed.Similarly, India imports oil from genetically modified soya bean for the past decade and genetically-modified canola has been imported for at least three years. China, however, has launched a comprehensive media campaign to back genetically modified crops. What is clear is that any policy changes with regards what may be regarded as 'GMO' virus vaccines must be evidence-driven.

Many questions - but only research will provide the answers.

DISCUSSION AND CONCLUSION

Developed countries have the economic resource to establish a comprehensive network of veterinarians (vital for the speedy diagnosis of FMDV infections in the field), building high containment laboratory facilities for detailed diagnosis (serotype/strain to inform vaccine deployment), similar high containment plant for vaccine production, tightly enforced standards in the trade of animals/ animal products plus high levels of vigilance at international borders. All of these factors lead to gaining the 'disease free without vaccination' status: the national herds are completely naïve. No anti-FMDV antibodies, no restriction on trade. However, the UK 2001 FMDV outbreak by the 'Pan Asian' strain vividly illustrates that food security is an international issue. Whilst there have been impressive advances in subunit/VLP vaccine technologies which could very well lead to improved disease security, in this review we argue that only an approach such as live, attenuated, vaccines that can be administered by inhalation (rather than injection), could effectively address the issue of wild life reservoirs of disease in Africa, S. America, Asia and the Indiansub-continent to ultimately eradicate FMDV which must be our ultimate goal.

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