Universal detection of foot and mouth disease virus based on the conserved VP0 protein [version 1; referees: awaiting peer review]

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Abstract

**Background**: Foot and mouth disease virus (FMDV), a member of the picornaviridae that causes vesicular disease in ungulates, has seven serotypes and a large number of strains, making universal detection challenging. The mature virion is made up of 4 structural proteins, virus protein (VP) 1 – VP4, VP1-VP3 of which form the outer surface of the particle and VP4 largely contained within. Prior to mature virion formation VP2 and VP4 occur together as VP0, a structural component of the pre-capsid which, as a result of containing the internal VP4 sequence, is relatively conserved among all strains and serotypes. Detection of VP0 might therefore represent a universal virus marker.

**Methods**: FMDV virus protein 0 (VP0) was expressed in bacteria as a SUMO fusion protein and the SUMO carrier removed by site specific proteolysis. Rabbit polyclonal sera were generated to the isolated VP0 protein and their reactivity characterised by a number of immunoassays and by epitope mapping on peptide arrays.

**Results**: The specific VP0 serum recognised a variety of FMDV serotypes, as virus and as virus-like-particles, by a variety of assay formats. Epitope mapping showed the predominant epitopes to occur within the unstructured but highly conserved region of the sequence shared among many serotypes. When immunogold stained VLPs were assessed by TEM analysis they revealed exposure of epitopes on the surface of some particles, consistent with particle breathing hitherto reported for some other picornaviruses but not for FMDV.

**Conclusion**: A polyvalent serum based on the VP0 protein of FMDV represents a broadly reactive reagent capable of detection of many if not all FMDV isolates. The suggestion of particle breathing obtained with this serum suggests a reconsideration of the FMDV entry mechanism.
Keywords
Foot-and-mouth disease virus, picornavirus, VP0, conserved sequence, serotype, diagnostic, epitope, vaccine

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**Abbreviations**

E.coli, *Escherichia coli*; FMDV, Foot and Mouth Disease Virus; HRP, horse radish peroxidase; IMAC, immobilised metal affinity chromatography; kDa, kilodaltons; P1, polypeptide 1; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Sf9, *Spodoptera frugiperda* cell line 9; SUMO, small ubiquitin-like modifier; VP0, virus protein 0; VP1, Virus protein 1; VP2, virus protein 2; VP3, virus protein 3; VP4, virus protein 4.

**Introduction**

Foot-and-mouth disease virus, FMDV, classified in the aphthovirus genus of the Picornaviridae family, causes vesicular disease in a number of cloven-footed species, typically cattle, sheep and pigs. In developed economies outbreaks of the disease in farmed herds are associated with significant financial loss while in less developed economies a loss of milk yield and fecundity have a direct community impact. Where possible the disease is controlled by vaccination and slaughter but the virus evolves constantly to evade host immunity leading to multiple strains. The antibodies raised during natural infection, or following vaccination, are restricted to predominant immunogenic regions on the virion surface and frequently have a very narrow spectrum of reactivity. Antibodies to virus non structural proteins are more cross reactive (e.g. 6,7) but are of limited value for vaccine research programs which are necessarily focused on only the structural proteins. Some broad-ranging detection agents such as recombinant integrin, a soluble form of the virus receptor, have been developed but as alternate virus receptors have been described these may not react with all isolates.

We have developed systems for the expression of recombinant empty FMDV capsids, principally for use as potential vaccines. Since these capsids contain no genome, PCR-based methods of quantitation are impossible and their characterisation relies extensively on antibody reactivity. However, strain divergence is such that antibodies suitable for the detection of a wide range of isolates can be difficult to source.

To generate an antibody reagent capable of detecting the majority of FMDV isolates we made use of the recent finding that fusion of the individual structural proteins of the virus, VP0, VP1 and VP3 to the small ubiquitin-related modifier (SUMO) protein as a carrier allows efficient expression and purification of each mature protein in *E.coli*. Of these, VP1 and VP3 exhibit extensive serotype variation making them unsuitable as the basis of a universal serum reagent while much of VP0 is less variable. VP0 is an assembly intermediate protein that is incorporated into virus particles and then cleaved autocatalytically into VP4+VP2 coincident with the incorporation of the RNA genome. Part of the VP2 sequence lies on the surface of the virus particle and is subject to antigenic variation, similar to that observed for VP1 and VP3, but sections of VP2, and all of VP4, lie on the inside of the particle, are not under immune selection, and are highly conserved across serotypes. Thus, VP0 is a suitable candidate for the generation of a serum with potentially broad cross-reactivity.

**Results and discussion**

To produce VP0 protein, the sequence encoding VP0 from FMDV strains O1 Manisa Tur69 one of the seven serotypes of FMDV worldwide, was fused in frame to the SUMO sequence in a T7 promoter driven bacterial expression vector (Figure 1A). Following transformation of an *E.coli* strain expressing the T7 polymerase, growth and induction, a SUMO-VP0 fusion protein with the predicted molecular mass of ~46kDa was identified in bacterial extracts (Figure 1B). Purification to homogeneity was achieved by virtue of the poly histidine tag present at the N-terminus of the SUMO domain and incubation with the SUMO specific protease Ulp1 produced two fragments representing the 11.5 kDa SUMO and ~33.5 kDa VP0 domains (Figure 1C). The free SUMO domain and any uncleaved SUMO-VP0 fusion protein were subsequently removed by adsorption to an IMAC resin and the resultant pure VP0 protein was used for immunisation.

A standard regimen of immunisation generated polyvalent sera in rabbits which were screened by western blot for reactivity with FMDV antigen expressed in insect cells. In these tests, VP0 is produced in insect cells as part of the processing and assembly reaction of the P1 precursor protein (cf. Figure 1A) and the cleaved mature capsid proteins assemble into empty capsids, otherwise called virus like particles. As the genomic RNA is not present, VP0 does not generally undergo further cleavage to VP4 and VP2. Reactivity was apparent with a band of 37kDa consistent with the apparent molecular mass on SDS-PAGE of VP0 synthesised by a range of FMDV serotypes. Antibody reactivity in the serum generated reacted well with empty capsids representing serotypes A Iran 7/13, O1 Manisa, O Turkey 05/2009, Asia1 Shamir (Figure 2A) and SAT2 Zim 7/83. To address if reactivity was also apparent on non-denatured antigen, the VP0 serum was also used as the primary antibody for flow cytometry of insect cells expressing each serotype following fixation and permeabilization. Reactivity was apparent with all samples (Figure 2B) but the intensity of staining was somewhat lower than might be expected from the strength of reaction to denatured antigen. However, as relatively little of the VP0 sequence used to generate the sera is exposed on the surface of the virus particle, a lower reactivity to assembled capsids is plausible. Reactivity was also apparent with individual empty capsids when the serum was used for immunogold transmission electron microscopy (Figure 2C). Interestingly only some particles bound gold suggesting a subset with exposed epitopes. Finally, the VP0 serum was used to probe western blots of sucrose gradient purified virus from infected cell supernatants. These samples contain largely VP2, not VP0, as authentic virus has undergone VP0 maturation but nevertheless residual VP0 was detected for many of the samples tested, as was the more major cleavage product VP2 (Figure 2D). Despite reaction with whole virus the serum showed no neutralising activity (unpublished study, Eva Perez), consistent with the principle neutralising determinants of FMDV being present in the VP1 protein. To identify the linear epitopes underpinning the breadth of the observed reactivity, epitope mapping was done using peptide microarrays of both O1
Manisa sequence used for VP0 expression and, to include a more phylogenetically distant serotype, the SAT2 Zim VP0 sequence. Multiple epitopes were apparent, but in the main they clustered in the amino-terminal VP4 region of the protein representing only ~10% of the polypeptide used as immunogen (Figure 3A). Specifically, the major epitopes spanned residues 8–18 near the amino terminus of VP4 and residues 28–40 further downstream. The major epitope in VP2 comprised residues 5–14 at the amino terminus with more minor reactivity towards the carboxyl terminus (Figure 3B). In the three-dimensional structure of FMDV O1 Manisa the identified VP4 epitopes lie in a disordered region where no clear polypeptide chain mapping is possible (Figure 4). The predominant VP2 epitope is visualised but is distended away from the main body of the protein while the minor VP2 epitopes at residues 145–152 and 200–207 lie within the main fold. The epitope mapping data would be consistent with poor antibody induction by the tightly folded β-sheet rich "jelly-roll" fold of the VP2 domain but ready antibody induction to the much less ordered and distended regions. A similar observation has been made for a related picorna-like virus, Israeli acute paralysis virus, following expression, immunisation and epitope mapping of the resulting serum18.

Virus diversity in the natural environment, such as that shown by FMDV, provides the impetus for the development of novel control solutions, such as new candidate vaccines. But a corollary is often that the reagents available to characterise such novel products, for example those developed to newly emerged strains, are limited. Our data show that a focus on the most conserved polypeptide sequence of the virus particle, coupled with efficient, non-denaturing purification of the requisite protein can provide an immunogen able of generating a serum that is cross reactive for many strains. Epitope mapping confirmed the basis of such cross reactivity was short conserved sequences predominantly at the N-terminus of VP4. The serum performed well on denatured antigen whether it was VP0 (empty capsids) or VP2 (virus) but titres were reduced on assembled forms of the same proteins consistent with most epitopes being inside the particle. The low but very specific labelling of particles observed by TEM could therefore represent deformed particles which expose the inner surface or the transient exposure of internal epitopes on the intact particle surface.

Figure 1. Construction, expression and purification of FMDV VP0. A. Cartoon showing the location of VP0 in the structural protein precursor P1 and its relation to the other mature structural protein VP1, VP3, VP4 and VP2 as well as its configuration as a SUMO fusion protein. B. Western blot, of bacterial expression cultures. Lanes 1 and 3, the SUMO vector alone, lanes 2 and 4, the SUMO-VP0 fusion protein. Lanes 1 and 2 were blotted with a FMDV serum and lanes 3 and 4 were blotted with a His tag serum. C. Coomassie blue stained gel of VP0 proteins purified from bacteria, Lanes 5 and 6 purified SUMO-VP0 before and after digestion with SUMO protease. The various individual proteins are indicated.
Figure 2. Test of VP0 serum by western blot and flow cytometry. A. Detection of VP0 by western blot of insect cells expressing recombinant empty capsids. Lane 1 – A Iran 9/97, Lane 2 - O1 Manisa, Lane 3 – O Turkey 5/09, Lane 4 – SAT2 Zim 7/83, Lane 5 – Asia1 Shamir, Lane 6 – control. B. Mean fluorescence of insect cells expressing the same serotypes as in panel A following staining by the VP0 serum and analysis by flow cytometry. C. Labelling of individual empty capsids by the VP0 serum and a gold conjugate. The empty capsids were of the A22 Iraq serotype and the field is typical of 6 micrograms recorded. Note only a subset of particles are labelled, some indicated. A lack of free gold particles suggests specific labelling even at high serum concentrations (1:50). The bar is 100nm. D. Detection of processed VP2 in native virus purified by sucrose gradient by western blot. The lane are 1 – O 1 Manisa, 2 – A 22 Iraq, 3 - Asia 1 Shamir, 4 – SAT2 Egypt. The cluster of bands around 25kDa on the stained gel (left panel) are the virus capsid proteins, including VP2, others are cellular contaminants. VP2 is identified by a VP2 monoclonal antibody (center panel) and by the VP0 serum (right panel). Markers to the left are in kilodaltons. Arrows to the right indicate VP0 (upper) and VP2 (lower).

Conclusions

The picornaviridae contain many examples where strain variation among family members is extensive. Our data suggest that the same principle of serum generation by highly purified VP0 could be used to generate a broadly reactive serum in these cases also.

Methods

Cloning and expression vector construction

The sequences encoding the VP0 section of the FMDV strains described were taken from the databases but synthesised de novo as dsDNA fragments (gBlocks - Integrated DNA Technologies, Leuven, Belgium). They were assembled into SUMO expression cassettes by ligation of a restriction fragment or by an infusion reaction such that fusion of the VP0 sequence was at the C-terminus of the SUMO domain. All vectors were sequence verified before use (Sanger sequencing service, Source Bioscience, Nottingham, UK). Expression generally used E.coli BL21 DE3 pLysS as described. A number of FMDV
sequences were used in the characterisation of the serum, either as purified virus grown in BHK21 cells or virus-like-particles expressed in insect cells including A Iran 9/97, A22 Iraq, Asia 1 Shamir, O Turkey 05/2009, O 1 Manisa, SAT2 Zim 7/83 and SAT2 4/2012 Egypt.

**SDS-PAGE analysis**

Samples of *E. coli* were resuspended directly in SDS loading buffer, boiled for 5 minutes, cooled, vortexed to shear bacterial DNA and spun briefly to remove insoluble murein (3 min, 13000 rpm bench microfuge). The equivalent of 50 microliters...
of original culture per well was applied to a 10% precast SDS-PAGE gel (Cat. No. 4561033 BioRad, Carlsbad, USA). Electrophoresis used a Mini-PROTEAN Tetra Cell (Cat. No. 1658004 BioRad, Carlsbad, USA) and was performed at 190V constant voltage for 30 minutes. Samples of insect cell lysate or purified virus were mixed with SDS loading buffer and prepared similarly. Gels were either stained with Coomassie blue or used for western blot.

Western blot analysis
Gels were transferred to Immobilon filters (Immobilon P Cat. No. IPVH00010 EMD Millipore) by semi-dry electro transfer using a HorizeBLOT 4M-R (Cat. No. WSE-4040 ATTO Corporation, Tokyo, Japan) operating at 12V for 60 minutes and the membrane blocked using 5% dried milk powder in PBS for 1 hour at room temperature or 4°C overnight. Following blocking, membranes were rinsed and washed twice in PBS + 0.5% Tween-20 (Sigma) (PBS-T). The primary rabbit antibody, produced as described herein, was diluted in PBS-T + 0.5% dried milk powder and incubated with the membrane for 1 hour at room temperature, followed by washing twice (15 minutes each) in PBS-T. A polyclonal anti-rabbit IgG conjugated to HRP (Cat. No. P0448, Agilent DAKO, Cheshire, UK) was diluted in PBS-T + 0.5% dried milk powder and incubated with the corresponding blot for 1 hour at room temperature. HRP detection used an ECL western blot detection reagent (EZ-Chemiluminescence Cat. No. K1-0170 GeneFlow Ltd, Lichfield, UK) and the filter was imaged while luminescent on a Syngene Chemi XL imager.

Immunogold labelling
Empty FMDV capsids, purified as described were adsorbed to carbon coated formvar grids by floating the grid on a droplet of sample for 5 minutes at room temperature. The grid was washed briefly in water and floated sequentially on a 1:5 dilution of the VP0 serum followed by a 1:50 dilution of a polyclonal anti-rabbit antibody conjugated to 10nm gold (Cat. No. G7402 Sigma-Aldrich, Poole, UK), each for 10 minutes at room temperature. The grids were washed with distilled water and counter stained with 2% uranyl acetate before examination using a Joel TEM operating a 200kV.

Flow cytometry
Insect cells (Sf9) expressing VLPs of the serotypes shown were grown in suspension cultures at 27 °C with shaking at 110rpm in EX-CELL® 420 Serum-Free Medium (Cat. No. 14420C Sigma-Aldrich, Poole, UK), harvested at 3 days post infection and fixed and permeabilized with BD Cytofix/Cytoperm (BD Bioscience). They were incubated with rabbit anti-VP0 serum (1:1000), washed and incubated with a polyclonal

Figure 4. Location of the recognised epitopes in the FMDV structure. On the left the protomeric building block of the virus icosahedral shell comprising one copy each of VP4 (yellow), VP2 (green) VP3 (orange) and VP1 (blue) is shown in side profile. To the centre and right VP1 and VP3 have been removed for clarity and the structure of VP4 and VP2 shown with a side or top profile with the identified epitopes coloured red. Where epitopes lie in an unstructured region they are indicated by a dashed red line which is for illustration only, the actual meander of the polypeptide in this region is unknown.
anti-rabbit IgG conjugated to FITC (Cat. No. F9887 Sigma-Aldrich, Poole, UK). Cells were analysed on a BD FACScan using CellQuest (Version 3.3 BD Bioscience) and the mean fluorescence intensity plotted.

Serum generation
Serum generation was outsourced to Covalab Cambridge, UK. VP0 sera were raised in 2 New Zealand female rabbits following a standard regimen of prime and two boosts with Freund’s complete and Freund’s incomplete adjuvant respectively (Standard Polyclonal Service Pack, 53 day protocol, Covalab Cambridge, UK). Each immunisation used 25 micrograms of purified VP0 protein and seroconversion was confirmed by western blot of a test bleed taken 2 weeks after the first boost. The VP0 serum has been registered with the Antibody Registry as Ian Jones; University of Reading, Cat# Anti-VP0 Man, RRID: AB_2732804.

Microarray epitope mapping
The serum was subject to epitope mapping at single amino acid resolution on commercial peptide arrays of the VP0 protein comprising 20mer peptides overlapping by 1 amino acid (PEPperMAP® Service, PEPperPRINT, Heidelberg, Germany).

Data availability
The VP0 serum described here has been registered with the Antibody Registry with the designation AB_2732804.

The data underlying this study is available from the Open Science Framework. Dataset 1: Wellcome Open Research https://doi.org/10.17605/OSF.IO/9CRN2

This dataset is available under a CCO 1.0 Universal license.

Competing interests
No competing interests were disclosed.

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