Universal detection of foot and mouth disease virus based on the conserved VP0 protein [version 1; referees: 3 approved with reservations, 1 not approved]

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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 polyvalent 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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Author roles: Loureiro S: Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation; Porta C: Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation; Maity HK: Formal Analysis, Investigation, Methodology; Perez E: Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation; Bagno FF: Formal Analysis, Methodology; Kotecha A: Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation; Ren J: Formal Analysis, Investigation, Writing – Original Draft Preparation; Stuart DI: Conceptualization, Funding Acquisition, Investigation, Writing – Original Draft Preparation; Hoenemann H: Formal Analysis, Investigation, Methodology; Serrano A: Formal Analysis, Investigation, Methodology; van den Born E: Conceptualization, Investigation, Supervision, Writing – Original Draft Preparation; Charleston B: Conceptualization, Funding Acquisition, Supervision, Writing – Original Draft Preparation; Jones IM: Conceptualization, Funding Acquisition, Project Administration, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This work was supported by the Wellcome Trust [101122]
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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How to cite this article: Loureiro S, Porta C, Maity HK et al. Universal detection of foot and mouth disease virus based on the conserved VP0 protein [version 1; referees: 3 approved with reservations, 1 not approved] Wellcome Open Research 2018, 3:88 (doi: 10.12688/wellcomeopenres.14655.1)

Abbreviations
E.coli, Escherichia coli; FMDV, Foot and Mouth Disease Virus; HRP, horse radish peroxidase; IMAC, immobilised metal affinity chromatography; kDa, kilodaltons; P1, polyprotein 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 integris, 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 used for 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 auto-catalytically 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 Tur/69 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 Zm 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 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

originally observed for rhinovirus and termed “breathing”21. Interestingly, for picornavirus examples where the breathing intermediate has been captured structurally, it is residues 1–50 at the N terminus of VP4 that are exposed22,23, consistent with the predominant targets of the serum generated here.
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

**Figure 3.** Epitope mapping of serum. **A.** Relative reactivity of the rabbit serum raised to the O1 Manisa VP0 on peptide arrays of O1 Manisa and SAT2 Zim. **B.** The major epitopes identified in the O1Manisa sequence shown as bold underlined text in the complete sequence. The junction of VP4 and VP2 is indicated by the green bar.
of original culture per well was applied of 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% 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 described11 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.](image)
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](https://doi.org/10.17605/OSF.IO/9CRN2)

This dataset is available under a CC0 1.0 Universal license.

**Competing interests**

No competing interests were disclosed.

**Grant information**

This work was supported by the Wellcome Trust [101122].

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Data Source
Loureiro and co-workers describe the expression of FMDV strain O1 Manisa Tur/69 protein VP0 ('uncleaved' VP4/VP2) as a His-tagged SUMO fusion protein in a bacterial expression system: Following purification using the His-tag, the SUMO fusion partner was removed by the SUMO-specific proteinase: the purified VP0 was then used to raise (rabbit) anti-VP0 antibodies. These polyclonal antibodies were then tested for cross-reactivity against proteins from other FMDV serotypes. I do not think the title (as it stands) is justified by the data presented in Figure 2, panels A and B (could data from naive Sf9 cells be included here?). 'Universal' is a reach. Naturally, not every FMDV serotype/strain needs to be tested, but more 'coverage' would give more confidence. On this point, could some indication as to the degree of conservation (across all FMDVs) of each amino acid shown in Figure 3 Panel B be given? These data are readily available. The structural analyses and discussion of the particle structure ('breathing') was very informative.

Is the work clearly and accurately presented and does it cite the current literature? Partly

Is the study design appropriate and is the work technically sound? Yes

Are sufficient details of methods and analysis provided to allow replication by others? Yes

If applicable, is the statistical analysis and its interpretation appropriate? Not applicable

Are all the source data underlying the results available to ensure full reproducibility? Partly

Are the conclusions drawn adequately supported by the results? Partly

**Competing Interests:** No competing interests were disclosed.
I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Referee Report 09 August 2018

doi:10.21956/wellcomeopenres.15957.r33570

Stephen Curry
Department of Life Sciences, Imperial College, London, SW7 2AZ, UK

This study aims to test the hypothesis that antisera raised against VP0 from one strain of FMDV (O1M) may serve as a detection reagent for all strains of the virus. The hypothesis is predicated on the assumption that the VP4 polypeptide segment within VP0 is relatively conserved between FMDV serotypes. The paper describes the generation of a bacterial expression vector for a his-SUMO-tagged VP0 protein, the use of this protein to generate antisera in rabbits, and characterisation of the antisera against a selected range of antigens.

In its present state the work represents an interesting set of observations, but there are some shortcomings in the experimental design and the reporting of the results that should be addressed. These are as follows:

1. The claim of 'universal detection' made in the title of the article is not supported because not all serotypes have been tested. No viruses of type C, SAT-1 or SAT-3 were included. Clearly an interesting range of serotypes has been used, giving some indication of the broad specificity of the antisera, but this is some way shy of 'universal'. Either the title should be modified or the missing serotypes included.

2. The evidence presented to suggest that FMDV particles may exhibit 'breathing' (as has been observed for other picornaviruses) consists of a single electron micrograph (Fig. 2C) for which there is no control (nor any attempt to discern conformational differences as a function of temperature). The claim of breathing motions is therefore highly speculative and should be removed from the abstract, if not from the entire paper. If it is to be retained, the authors should cite the elegant 1994 study on reversible exposure of VP4 and VP1 termini in polioviru.

3. The expression and purification of the VP0 antigen is not described in the paper. The method should be included and it would be useful to include an indication of the yield of purified protein and some estimate of its solubility (as a benchmark for anyone seeking to build on this work).

4. In Fig. 2A it is surprising to me that the detection of VP0 from 01 Manisa is so weak because this is the same strain as the antigen used to generate the antisera. I confess I thought at first that the blot lanes had been mislabelled. The authors should comment on the variability of the detection of different strains – I don't think the claim that the antisera 'reacted well' with the 5 VP0s tested is sufficient. The nature of the sample included in the control should also be stated – was this an non-FMDV VP0?

5. In Fig 2B there is a very different pattern of reactivity compared to Fig. 2A. I presume this is in part because of the different conformational state of the antigen being detected. This is presumed to be 'non-denatured' but it would be useful if the authors would explicitly state the assumptions about the state of the FMDV antigens in fixed, permeabilised cells. Much will be intact virions but are there also assembly
intermediates and some denatured material?

6. In the legend to Fig. 2C it is revealed that the type A strain used is A22, which is different to the A strains used in the experiments in panels A and B. It would be helpful to mention this in the body of the text where the result is described.

7. On page 4, it is stated that the authors have achieved "efficient, non-denaturing purification of the requisite protein" (i.e. VP0) - but no data is shown to support this. (See point 3 above).

8. On the same page a claim is made for "very specific labelling of particles observed by TEM" but there were no controls or comparators presented to support this. The claim should be removed or appropriate controls presented.

9. Fig. 2B - how many independent measurements were made to determine the mean fluorescence for each antigen type? This should be stated and an error estimate provided.

10. Fig. 2D - what is the source and specificity (i.e. which FMDV strain) of the anti-VP2 antibody used in the middle panel?

11. Fig. 3a. It would be useful to indicate the boundary between VP4 and VP2. The reader would also benefit from a VP4 sequence alignment of the FMDV strains included in this study – and/or a quantitative analysis of the sequence conservation in VP4.

References

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Referee Expertise: Structural virology, picornaviruses
I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Trevor Sweeney
Division of Virology, Department of Pathology, Addenbrooke’s Hospital, University of Cambridge, Cambridge, UK

Loureiro et al. describe the reactivity of a rabbit serum preparation from animals injected by bacterially expressed and purified VP0 protein from foot-and-mouth disease virus (FMDV). The authors show cross reactivity with a number of FMDV serotypes but the serum is non neutralising. From epitope mapping and transmission electron microscopy analysis using gold labelled antibodies the authors suggest that the region of VP0 recognised by the serum is only exposed some of the time in virus like particles and that this may reflect 'breathing' of the particles as described for other picornaviruses but not FMDV.

Overall the work is clearly presented and well written and the experiments are well performed. There are a number of areas however in which the text could be expanded to help the reader appreciate the significance of the results.

Introduction, first paragraph, sentence 3: 'Where possible the disease is controlled by vaccination and slaughter but the virus evolves constantly to evade host immunity leading to multiple strains'. This sentence could be modified by splitting into two and removing the 'but'. Viral outbreaks can be controlled by vaccination and slaughter despite viral evolution, the sentence as currently written suggests this is not the case.

Results and Discussion, Figure 1: Is there a reason why the VP0 signal is so disperse in lane 6 compared to the that in lane 4, as both samples were run on SDS-PAGE gels before different subsequent treatments?

Results and Discussion, Figure 2: 2A, the additional bands in lanes 3 and 4 are overlooked in the text and should at least be noted. 2C, is the remaining signal (aside form the obvious capsids) in this microgram from denatured protein in the samples prep or a result of the adsorption procedure?

Results and Discussion: last paragraph, the results/discussion would benefit from a description of the current understanding of FMDV entry and how the presence of breathing would alter our understanding of this.

Title and abstract: The title states 'universal detection' but not all serotypes have been tested. For the same reason the 'if not all' should be removed from the first sentence on the Abstract Conclusion.

Is the work clearly and accurately presented and does it cite the current literature? Yes
Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Referee Expertise: Molecular virology, virus replication, protein translation regulation, biochemistry

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Graham J Belsham

DTU National Veterinary Institute, Technical University of Denmark, Kalvehave, Denmark

Loureiro et al., have expressed the foot-and-mouth disease virus capsid component VP0 in a bacterial expression system and generated polyclonal antisera against it. Note VP0 is cleaved during particle assembly to the mature VP4 and VP2 products. The anti-VP0 antisera has then been characterized for its ability to detect FMDV in a variety of assays. Due to the relatively high conservation of the N-terminus of VP0, it is not surprising that the antiserum recognizes multiple serotypes of FMDV. Indeed, monoclonal antibodies that recognize all serotypes of FMDV have been described previously\(^1\)-\(^3\). These reagents have formed the basis for the pan-FMDV antigen detection systems used in lateral flow devices used in “penside” tests. The Pirbright Institute had a major role in the development of these tests and so it is very surprising that this information is not mentioned anywhere. Such monoclonal antibodies recognize epitopes within VP2\(^1\),\(^3\).

Major points

1. The text and Fig. 1 legend refer to the structural protein precursor P1, this is incorrect. For FMDV, the structural protein precursor is P1-2A (in the enteroviruses (like poliovirus) the precursor is P1) and it would be better if both Fig.1 and the text were modified to indicate this. It would also be useful if the His-tag within the SUMO fusion protein was also indicated.

2. In the Results and Discussion section, it is indicated that the VP0 was purified to homogeneity but this is not actually shown- why not? Fig 1C only shows the products prior to the removal of the
2. His-tagged SUMO. Even here, the VP0 product appears to migrate as a rather heterogeneous band- why is this?

3. The text indicates that VP0 cleavage to VP4 and VP2 does not generally occur in the absence of encapsidated RNA. However, as some of these authors have shown, this is not actually the case for FMDV empty capsid particles\(^4\), at least when produced in mammalian cells (maybe this is different in the insect cell system which would be interesting).

4. The text indicates the anti-VP0 antibody reacts well with the empty capsids from 5 different serotypes of FMDV when analysed by Western blot (see Figure 2A). However, in my view, good reactivity is only seen with 2 of the strains (O Turkey (lane 3) and SAT 2 Zim (lane 4), I think the text needs modifying. In contrast, the reactivity of the serum with insect cells expressing the empty capsids of the different serotypes shows very weak reactivity with the O Turkey and SAT 2 Zim samples (lanes 3 and 4, Fig 2B). This does not match the text which needs to be modified. Is it significant that the strains poorly recognized in the flow cytometry assays are those best recognized in the Western blot? The authors should comment.

5. In Fig 2C, gold labeling of A22 empty capsids is shown. The text indicates “only a subset of particles are labeled”, this is undoubtedly the case. Unfortunately, there are no controls here, e.g. with a different antiserum, thus I am not sure how the authors can justify the statement that this is “very specific labeling of particles” ((P.4). Indeed, it also seems impossible to know whether the particles that are labeled are damaged particles or not (as mentioned on P.4) and I think the Discussion of virus “breathing” is excessive. The earlier work on rhinovirus “breathing”\(^5\) would suggest exposure of VP4 was relatively frequent but that does not appear to be the case here. Thus the sentence in the Conclusion of the Abstract (P.1) stating “The suggestion of particle breathing obtained with this serum suggests a reconsideration of the FMDV entry mechanism.” seems far from justified; indeed this is not even mentioned in the main text and should be deleted from the Abstract.

6. In Fig 2D, it is surprising that the anti-VP2 monoclonal antibody apparently fails to recognize the A22 VP2. Has this been observed previously? A positive control for the presence of FMDV capsid proteins in the samples would have been useful. It is also unfortunate that there are no negative control lanes (e.g. poliovirus empty capsids) within Fig 2D, why not? It would be interesting to know if the anti-VP0 antisera recognizes VP4 on Western blots. This should have been apparent in the right hand panel of Fig 2D since purified native virus particles were used but the VP4 may migrate faster than the 10kDa marker and thus maybe was not detected on this gel, a higher percentage gel would have been helpful. This should be determined.

7. I am not convinced it is possible to draw any conclusions about the nature of the recombinant VP0 structure based on previous data for the whole P1-2A precursor, I think the text needs modifying.

Minor points

1. There are many minor errors and inconsistencies in the text that should have been corrected by careful reading before submission.

2. In Fig 3 B, the sequence of VP0 is shown here with an N-terminal Met (M) residue. This is incorrect; the VP0 sequence is preceded by the Leader protease in the viral polyprotein and in the SUMO fusion, the VP0 sequence is preceded by the SUMO sequence so there is no need for an initiator methionine residue. I think it would be better to show a comparison of the VP0 sequences from O1 Manisa and the SAT 2 Zim as used for the peptide arrays to show the regions with high similarity in the VP0 of these viruses.

References

1. Yang M, Clavijo A, Suarez-Banmann R, Avalo R: Production and characterization of two serotype


Is the work clearly and accurately presented and does it cite the current literature? 
Partly

Is the study design appropriate and is the work technically sound? 
Yes

Are sufficient details of methods and analysis provided to allow replication by others? 
Yes

If applicable, is the statistical analysis and its interpretation appropriate? 
Not applicable

Are all the source data underlying the results available to ensure full reproducibility? 
Partly

Are the conclusions drawn adequately supported by the results? 
Partly

**Competing Interests:** No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.