Identification of novel epitopes in serotype O foot-and-mouth disease virus by in vitro immune selection

Mana Mahapatra, Sasmita Upadhyaya and Satya Parida*

Abstract

Foot-and-mouth disease virus (FMDV) displays various epitopes on the capsid outer surface. In addition to the five neutralizing antigenic sites, there is evidence of the existence of other, yet unidentified, epitopes that are believed to play a role in antibody-mediated protection. Previous attempts to identify these epitopes revealed two additional substitutions at positions VP2-74 and -191 (5M2/5 virus) to be of antigenic significance. However, complete resistance to neutralization was not obtained in the neutralization assay, indicating the existence of other, undisclosed epitopes. Results from this study provides evidence of at least two new neutralizing epitopes involving residues VP3-116 and -195 around the threefold axis that have significant impact on the antigenic nature of the virus. These findings extend our knowledge of the surface features of the FMDV capsid known to elicit neutralizing antibodies, and should help with rational vaccine design.

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals, with the primary domesticated hosts being cattle, buffalo, sheep, pigs and goats. The disease spreads mainly by direct contact, contaminated articles (fomites) and also by the airborne route, and is widespread across the world, especially in Africa and Asia. In addition to circulation of FMD in endemic regions, there are occasional incursions into countries that are normally disease-free, e.g. in the UK in 2001 and in Japan in 2011, resulting in huge economic losses because of strict regulation on international trade of animals and animal products. The causative agent, FMD virus (FMDV), is a non-enveloped, single-stranded, positive-sense RNA virus in the genus Aphthovirus that belongs to the family Picornaviridae. It exists as seven immunologically distinct serotypes (O, A, C, Asia 1, SAT 1, SAT 2 and SAT 3), with numerous subtypes within each serotype. Vaccination with one serotype does not confer protection against infection with another serotype, sometimes not even against other subtypes within the same serotype [1–4]. Thus antigenic mis-match is an important reason for vaccine failure contributing to disease control challenges. Of the seven serotypes, type O is the predominant and causes more than 60% of outbreaks worldwide [5, 6]. The viral genome is about 8.5 kb and contains a single, large, open reading frame that encodes four capsid proteins (VP1–4), as well as a total of nine additional mature, non-structural proteins. Sixty copies each of the structural proteins form the capsid. The viral proteins VP1–3 are exposed on the outer surface of the virus particle whereas VP4 is internal. The outer capsid proteins VP1–3 determine both the reactivity of virus-neutralizing antibodies and the ability of the virus to interact with its receptors on host cells.

Conventional FMD vaccines consist of chemically inactivated purified whole-virus preparations that are used worldwide [3]. Although the mechanism of protection afforded by FMDV vaccines is not fully understood, the critical role of neutralizing antibodies in protection induced by FMD vaccination is well established [7]. Over the years, several studies have been carried out to identify the targets on the virus surface for the binding of neutralizing antibodies. In particular, neutralizing monoclonal antibodies (mAbs) have been used as powerful tools for identifying the amino acids that are within different antigenic sites, mainly by sequencing mAb-resistant (mar) mutants. However, with advances in technology, other methods for epitope prediction have become available. The main examples use either the known crystal structure of the capsid or the amino acid sequences of the viral capsid [8–10]. In addition, polyclonal antibodies have also been used to characterize epitopes [11–14]. Based on neutralizing mAb-binding studies, several antigenic sites have been identified in FMDV and serotype O has been most extensively studied, resulting in the identification of five neutralizing antigenic sites (sites 1–5) on the surface of type O virus. Of these three sites, 1, 3 and 5 are located in VP1 whereas site 2 includes residues in VP2 and site 4 includes part of VP3. Site 1 is linear and trypsin sensitive,
whereas all other antigenic sites are conformational and trypsin resistant. For serotype O viruses, the G–H loop and carboxy (C)-terminus of VP1 contribute to site 1, with critical residues that affect antibody binding at positions 144, 148, 150 and 208. Critical amino acids within site 2 are at positions 70–73, 75, 77 and 131 in VP2, while changes in VP2-79 and VP2-134 were reported to affect binding of site 2 mAbs [15]. For site 3, the key residues are at 43 and 44 within the B–C loop of VP1, while only one critical residue, VP3-58, has been identified for site 4. The fifth antigenic site, including residue 149 of VP1, is probably formed by interaction of the VP1 G–H loop region with other surface amino acids [16–21].

In the case of O1 Kaufbeuren (O1K), a quintuple mutant virus was made by successive single-step selection with mAbs directed at neutralizing antigenic sites 1–5 [17, 19]. This mutant virus was reported by Dunn et al. to resist neutralization by bovine polyclonal sera raised against the parent virus [22]. Furthermore, these authors used this quintuple mutant in guinea pig experiments to explore the contribution of these neutralizing epitopes to the protection conferred by inactivated vaccines. In their study, guinea pigs were immunized with either wild-type (wt) or mutant 146S antigen as inactivated whole-virus vaccines or hyperimmune sera raised against the vaccine antigen, and challenged with virulent wt parent and mutant viruses. It was expected that none of the animals vaccinated with the mutant antigen would be protected as the epitopes in each antigenic site were mutated, and therefore antibodies to these epitopes would not be produced following immunization. Contrary to this expectation, all animals were protected indicating that (1) the antigenic sites could be much larger than the epitopes recognized by individual mAbs, since changing single residues does not necessarily modify the entire antigenic site; and (2) the existence of other, undisclosed protective epitopes in FMDV. The identity of these was explored in a previous study, using a reverse genetics approach. Substitutions at two capsid residues, VP2-74 and VP2-191, were reported to have significant impact on the antigenicity of the virus [23]. In that study a recombinant virus (5M2/5) was made that contained substitutions at the critical amino acid residues of the five neutralizing antigenic sites along with two additional substitutions at positions VP2-74 and -191. Serological characterization of this recombinant virus, 5M2/5, exhibited greater resistance to neutralization (a further 20% reduction) with both O1K guinea pig and O1 BFS bovine antiserum than a virus that was engineered to include only substitutions at the five known antigenic sites (5-site mutant virus, 5M). As complete resistance to neutralization was not observed, it was concluded that other, unidentified epitopes may exist on the
surface of the capsid that could be the cause of the residual neutralization observed with serology. In the present study, attempts were made to identify additional capsid amino acid residues that could have antigenic significance. Results from this study identified two capsid residues in the VP3 protein that, to date, have not been reported as having an effect on the antigenicity of serotype O FMDV, and could be part of new epitopes. These results extend our knowledge on the surface features of the FMDV capsid known to elicit neutralizing antibodies, and could also help development of more broadly cross-reactive vaccines.

In a previous study we made a recombinant virus (rO1K) containing the full-length FMDV O1K (wt) cDNA. Another recombinant virus (5-site mutant, 5M) was made using this cDNA clone in which all five neutralizing antigenic sites were mutated (Fig. 1a); the capsid-coding region of this virus is identical to the capsid sequence of the quintuple mutant made by Crowther and colleagues [19]. A third recombinant virus was also made in this study using the 5M cDNA clone, where two additional substitutions, at positions VP2-74 and -191 (5M2/5) were added (as described in [23]) (Table 1a). Antisera were raised in five cattle against rO1K viral antigen in the animal isolation unit at the Pirbright Institute, UK (with approval of the institute ethical committee), following the method described previously [24]. Serum samples collected 21 days post-vaccination (neutralizing antibody titres ranging log$_{10}$ 2.45–3.18) were used subsequently in this study. Serum from a single cow (no. 395) was used for selection of mutant viruses, whereas both serum from this animal (O1K-BS-395) and pooled sera from all five animals (O1K-BS) were used in serological assays (virus neutralization test) to characterize the mutant viruses. Viruses rO1K and 5M2/5 were incubated in sub-

<table>
<thead>
<tr>
<th>Antigenic sites/mutants</th>
<th>Site 1</th>
<th>Site 2</th>
<th>Site 3</th>
<th>Site 4</th>
<th>Site 5</th>
<th>Additional substitutions</th>
</tr>
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<tr>
<td>Viral protein positions</td>
<td>VP1</td>
<td>VP1</td>
<td>VP1</td>
<td>VP2</td>
<td>VP1</td>
<td>VP1</td>
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<tr>
<td>rO1K</td>
<td>R</td>
<td>L</td>
<td>V</td>
<td>S</td>
<td>T</td>
<td>E</td>
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<td>R</td>
<td>A</td>
<td>N</td>
<td>K</td>
<td>V</td>
</tr>
<tr>
<td>5M2/5</td>
<td>K</td>
<td>R</td>
<td>A</td>
<td>N</td>
<td>K</td>
<td>V</td>
</tr>
</tbody>
</table>

Table 1. (a) List of O1 Kaufbeuren mutant viruses used in this study and their associated amino acid substitutions.

Positions different from the rO1K virus are shaded.

<table>
<thead>
<tr>
<th>Viral protein</th>
<th>nt position</th>
<th>Codon change</th>
<th>aa position</th>
<th>aa change</th>
<th>Passage</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP3</td>
<td>584</td>
<td>GAC-GGC</td>
<td>195</td>
<td>D-G</td>
<td>P9</td>
</tr>
<tr>
<td>VP1</td>
<td>140</td>
<td>CAA-CGA</td>
<td>47</td>
<td>Q-R</td>
<td>P9</td>
</tr>
<tr>
<td></td>
<td>629</td>
<td>AAA-AATA</td>
<td>210</td>
<td>K-I</td>
<td>P12</td>
</tr>
</tbody>
</table>

Table 1. (b) Capsid amino acid (aa) residue changes observed in mutant virus rO1K-S-P25.

Nucleotide (nt) changes in the codon are underlined.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Viral protein</th>
<th>nt position</th>
<th>Codon change</th>
<th>aa position</th>
<th>aa change</th>
<th>Passage</th>
</tr>
</thead>
<tbody>
<tr>
<td>5M2/5 S-P25$</td>
<td>VP2</td>
<td>233</td>
<td>TGC-TAC</td>
<td>78</td>
<td>C-Y</td>
<td>P3</td>
</tr>
<tr>
<td></td>
<td>VP3</td>
<td>163</td>
<td>CTC-CTG</td>
<td>55</td>
<td>T-M</td>
<td>P24‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>173†</td>
<td>GAG-GGC</td>
<td>58</td>
<td>V-A</td>
<td>P14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>346</td>
<td>GAC-AAC</td>
<td>116</td>
<td>D-N</td>
<td>P6</td>
</tr>
<tr>
<td>5M2/5-P25$</td>
<td>VP1</td>
<td>486*</td>
<td>TCC-TCT</td>
<td>162</td>
<td>S-S</td>
<td>P24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>203</td>
<td>AGC-AGC</td>
<td>68</td>
<td>M-T</td>
<td>P23‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>223</td>
<td>GCT-AGT</td>
<td>75</td>
<td>A-T</td>
<td>P14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>234*</td>
<td>GCA-GAT</td>
<td>78</td>
<td>D-D</td>
<td>P19</td>
</tr>
</tbody>
</table>

Table 1. (c) Capsid amino acid (aa) residue changes observed in 5M2/5 mutant viruses passaged in the presence or absence of sera.

The nucleotide (nt) changes in the codon are underlined.

*indicates synonymous change.
†indicates change observed in epitope as identified in the quintuple mutant.
‡indicates mixed base observed at respective passages that became fixed in subsequent passage.
§indicates viruses passaged further up to P30 to confirm the changes that appeared very late (P23 and P24).
neutralizing concentrations of antiserum for 30 min and then added to confluent pig kidney cells (IBRS2). The virus/antiserum mixture was left on the cells for 1 h for adsorption, after which the inoculum was removed and the cells were washed at least twice with phosphate buffered saline (PBS) before cell culture medium was added. The cells were incubated at 37°C and checked under a microscope every 6–8 h for the appearance of FMDV-specific cytopathic effect (CPE). As a control, the same virus was propagated in the absence of any treatment but under otherwise similar conditions and up to the same passage level.

Following passage in the presence of anti-FMDV serum, reduced viral fitness in cell culture was observed. There was about 5% CPE after 24 h incubation; by 72 h post-incubation there was about 30–40% CPE whereas in the case of parent viruses complete CPE was observed by 12–18 h post-infection. This process was continued for seven passages, with a gradual increase in escape from neutralization following each passage, and on passage 8 about 90% CPE was observed by 48 h post-infection (data not shown). In the following passage, the level of serum in the virus/antiserum mixture was doubled. A total of 25 passages was carried out, generating a total of four viruses, two following passage in the absence of antiserum (rO1K-P25 and 5M2/5-P25) and a further two passaged in the presence of antiserum (rO1K-S-P25 and 5M2/5 S-P25) (Fig. 1a).

**SEROLOGICAL CHARACTERIZATION OF MUTANT VIRUSES**

As the main aim of this study was to quantify the reduction in neutralization by viruses containing mutations in the capsid surface of FMDV, it was crucial to determine the virus-neutralizing antibody (VN) titre of the sera against all mutant viruses at a fixed virus dose (100 TCID₅₀), for which a 2D micro-neutralization test was carried out using five different doses of the virus encompassing 100 TCID₅₀ for this purpose. The resultant VN titres at each virus dose were used to calculate the serum titre at 100 TCID₅₀ by regression analysis, and the results are shown in Fig. 1 (b and c).

The VN titre of the rO1K-P25 virus that was passaged in the absence of serum was similar to the parent virus (rO1K), whereas for the rO1K-S-P25 virus that had been passaged in the presence of antiserum a significant reduction (~25%) in VN titre was observed (Fig. 1b). Similarly in
the case of the multiple mutant virus 5M2/5-P25 (passaged in the absence of antiserum) a further 5% reduction in VN titre (compared to 5M2/5 virus) was observed, although this was not statistically significant. In contrast 5M2/5 S-P25 exhibited a further 25–30 % reduction in VN titre (Fig. 1c) taking it up to a total of 90 % reduction compared to the parent rO1K virus.

**GENETIC CHARACTERIZATION OF MUTANT VIRUSES**

The capsid-coding regions of the mutant viruses at P25 were amplified by RT-PCR, sequenced on both strands and the sequence data assembled as described previously [25]. Sequence analysis of virus rO1K–P25 did not reveal any changes at the nucleotide (nt) level. This is not surprising, as this virus was first isolated in 1966, has been used in the laboratory for a long time and has also been passaged in cell culture many times. However, when the capsid sequence of rO1K-S-P25 virus was analysed, three non-synonymous changes were observed, two in VP1 (residues 47 and 210) and one in VP3 (residue 195) (Table 1 b); all of these residues are surface exposed (Fig. 2 a and b). One of these residues, VP1-47, is located near antigenic site 3. Residues 43–45 and 48 in VP1 have previously been identified as part of antigenic site 3 in the case of serotype O FMDV [16, 17, 26]. Although residue VP1-47 has never been reported as being critical by mar-mutant studies in serotype O FMDV, it could be part of antigenic site 3 as it is located very close to

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**Fig. 3.** (a) 3D structure (external surface) of the O1K (reduced) FMDV showing a potential new antigenic site within the circle. Changes in amino acids in the quintuple mutant (5M) forming the five neutralizing antigenic sites (as determined by murine mar-mutant studies) in the central protomer (shown in white) are shown in blue. Critical residues identified using bovine mAbs [20] are shown in orange. Changes in residues introduced by reverse genetics in a previous study [23] are shown in green. Changes identified in this study in viruses rO1K-S-P25, 5M2/5-P25 and 5M2/5 S-P25 are shown in purple, cyan and red, respectively. The threefold axis is indicated by a black arrow. (b) Schematic diagram (not to scale) showing the distance (in Å) between the capsid residues at the proposed antigenic site.
Table 2. Capsid amino acid residues at different positions in the proposed antigenic site in different serotypes

<table>
<thead>
<tr>
<th>Serotype</th>
<th>No. of sequences analysed</th>
<th>VP2-188</th>
<th>VP2-191</th>
<th>VP3-116</th>
<th>VP3-195</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>433</td>
<td>T</td>
<td>T&gt;N&gt;S</td>
<td>D</td>
<td>D&gt;E&gt;N</td>
</tr>
<tr>
<td>A</td>
<td>391</td>
<td>T</td>
<td>S/N/D/T</td>
<td>D/E</td>
<td>A/V</td>
</tr>
<tr>
<td>C</td>
<td>27</td>
<td>T</td>
<td>T</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Asia-1</td>
<td>40</td>
<td>T</td>
<td>T</td>
<td>A</td>
<td>G</td>
</tr>
<tr>
<td>SAT-1</td>
<td>46</td>
<td>T</td>
<td>T</td>
<td>N</td>
<td>T</td>
</tr>
<tr>
<td>SAT-2</td>
<td>44</td>
<td>T</td>
<td>Q</td>
<td>H</td>
<td>T</td>
</tr>
<tr>
<td>SAT-3</td>
<td>23</td>
<td>T</td>
<td>N/S/T</td>
<td>Q</td>
<td>T</td>
</tr>
</tbody>
</table>

other previously identified residues. In addition, changes in this region (VP1 40–60) were previously reported in the case of serotypes C and O FMDV following immune selection using antiviral polyclonal sera [13, 14]. Similarly, a change in VP1-210, which is part of antigenic site 1, was observed. A change in the C-terminus of VP1 (VP1-209) was also observed in FMDV O1 Caseros following immune selection [11]. Interestingly residue 210 at the C terminus of VP1 actually lies close to site 3; in fact, it protrudes out of the original protomer and usually lies against the groove in the biologically adjacent protomer (Fig. 3 a). The third change noted was from an aspartic acid (D) to glycine (G) at VP3-195 that was observed at passage 9. This residue is located close to the threefold axis and only 6.5 Å apart from VP2-188 that was identified as an epitope using bovine mAb [20]. This residue (VP3-195) has been reported to be an important part of an epitope in serotype A (A10) virus [27]. Therefore, this could be part of a new epitope in serotype O FMDV that is targeted during immune escape along with antigenic sites 1 and 3. However, the impact of the change in this residue on resistance to neutralization is not clear from the findings of this study, as the reduction in VN titre (~25%) by the mutant virus, rO1K-S-P25, could have been the cumulative effect of all three substitutions observed in this virus. Targeted mutagenesis studies involving a cDNA clone could confirm this observation.

Analysis of the sequence data of the 5M2/5-P25 virus that was passaged without antiserum revealed three nt changes, all in the VP3 coding region (Table 1c). Of these changes, that at VP3-78 was synonymous whereas the other two (VP3-68 and -75) were non-synonymous and surface exposed (Fig. 3a–b). The nt change for the synonymous change was in the third base of the codon, whereas in the case of the non-synonymous changes the nt changes were either in the first or second base (Table 1c). The VP3-75 is located very close to VP3-58, which is involved in antigenic site 4, whereas residue VP3-68 is located close to VP3-195, which was identified in the rO1K-S-P25 virus. The antigenic significance of these capsid residues is unlikely to be high, based on a reduction of only ~5% in VN titre.

A total of five nt changes (one each in the coding regions for VP1 and VP2, and three in VP3) were observed in virus 5M2/5 S-P25 that was passaged in the presence of antisera (Table 1c). Of these, the nt change in the codon for residue 162 of VP1 was a synonymous change whereas the remaining four were non-synonymous changes, with three substitutions in VP3 and one in VP2 (Fig. 2c and d). Of these, the change at residue VP3-55 appeared very late, at P24 as a mixed sequence that became fixed at P25. This residue is located close to VP3-58, which is within antigenic site 4, although it is not exposed on the surface (Fig. 2d). The change at residue 78 of VP2 appeared very early at P3; this epitope was reported to be within antigenic site 2 as identified using bovine mAbs [20]. This is not surprising, as epitopes present within antigenic site 2 have been reported to be dominant within the polyclonal response of serotype O-vaccinated animals, and mutations in this area resulted in significant reduction in neutralizing antibody titres [28]. A valine (V) to alanine (A) change was observed in VP3-58 within antigenic site 4. In the parent rO1K virus this was a negatively charged residue, glutamic acid, and was changed to a hydrophobic residue, valine, in the 5-site mutant virus. Interestingly, at passage 14, the residue at this position (VP3-58) changed to another hydrophobic residue, alanine, and did not revert back. As both the residues are hydrophobic in nature, and also that there is little difference in the size of these residues, it is possible that the impact of this change on the antigenicity of the virus is not very profound. Mutagenesis studies involving a cDNA clone could resolve this. The final change observed at P6 was in VP3-116, which is located at the threefold axis (Fig. 3a) and could be part of a new epitope not previously reported for any FMDV serotype.

A total of six capsid residues involving four antigenic sites (1–4) were identified as having antigenic significance in this study. No change in antigenic site 5 (involving VP1-149 of the G–H loop) was observed in this study, which is not surprising since a total of four changes were introduced into the G–H loop of VP1 (VP1-138, -148, -149 and -150) of the mutant virus, 5M2/5, which was sufficient to abrogate binding of antibodies to the epitopes in the G–H loop. Therefore, other capsid regions of antigenic significance with less modification were targeted during the immune escape process. Residues VP3-116 and -195 are located at the threefold axis and are very close to residues VP2-188 and -191. VP3-116 is located at the centre and equidistant from VP3-195 and VP2-188 and -191 (Fig. 3b). Together, these four residues could form an antigenic site with VP3-116 at the centre surrounded by VP2-188, -191 and VP3-195. It would have
been very interesting to test this by using mAbs that bind in this region; unfortunately, no such mAbs exist in our collection or to the best of our knowledge in other FMD laborato-
ries. Furthermore, the capsid-coding regions of different
serotypes of FMDV were retrieved from the NCBI database
(as of 1 February 2019) and aligned to check the level of
conservation of these four residues in different serotypes
(Table 2). Analysis of the sequence data revealed a high level
of conservation in most critical residues previously identified
by mar-mutant studies, indicating the importance of these
residues in the viral life cycle. Similar to this observa-
tion, a high level of conservation was observed in these four
residues at the proposed antigenic site; VP2-188 was
completely conserved in all the serotypes, followed by VP3-
116 which was completely conserved in six out of seven
serotypes except serotype A, where it was always a nega-
tively charged residue, either a glutamic acid or an
aspartic acid.

In conclusion, changes in most antigenic sites (1–4), except
site 5, were identified in this study which either individually
or in combination, underpin the observed antigenic pheno-
types in viruses generated by immune selection, emphasizing
the importance of neutralizing antigenic sites during
immune escape. This study also provides evidence of new
neutralizing epitopes involving capsid residues VP3-116 and
-195 that have not previously been identified in mar-
mutant studies. The findings here extend our understanding
of the antigenic role of various features of the FMDV capsid
surface that can be used to predict the importance of evolu-
tional changes in circulating viruses, and thereby can help
in the design of better cross-reactive vaccines.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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