Appendix 43

Use of monoclonal antibodies to identify and map new antigenic determinants involved in neutralisation on FMD viruses type SAT 1 and SAT 2

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Abstract:

The antigenic structure of foot-and-mouth disease viruses (FMDV) of the SAT types was little investigated. Monoclonal antibodies (MAbs) provide a great help to these studies; they also may be valuable diagnostic tools and ideal reagents for highlighting antigenic differences, provided that the target sites and their implication in protection are known. This paper reports on the identification and mapping of neutralising sites in FMDV type SAT 1 and 2 using new panels of MAbs.
The approach adopted to this purpose was the selection of viral mutants resistant to MAb neutralisation and the subsequent detection of amino acid substitutions responsible for mutation.
For FMDV type SAT 1, results led to the identification of four independent antigenic determinants: nine MAbs identified two separate linear epitopes in the GH loop of VP1; five MAbs identified a new site, composed by epitopes that may involve two residues simultaneously, one in VP3 (positions 135 or 71 or 76) and one in VP1 (positions 179 or 181); four MAbs reacted with a conformation-dependent site involving positions VP1 181 and VP2 72; a further new site was detected by two MAbs mapping at position 111 of VP1.
For FMDV type SAT 2, three strongly neutralising MAbs identified two independent linear sites, one mapped at VP1 C-terminus (residue 210) and the other within the VP1 G-H loop (position 154). The latter may be associated with a further mutation at VP2 79.
In conclusions, the G-H loop of VP1, known as a major antigenic determinant in FMDV types O, A, C and Asia1, is an immuno-dominant site also in SAT 1 and SAT 2 serotypes. The VP1 C-terminus has also been confirmed as an independent linear site in FMDV SAT 2. Interestingly, three conformational sites detected in SAT 1 virus were never described before: they could represent new major determinants in FMDV or denote a different antigenic structure in the SAT serotypes.

Introduction:

Foot-and-mouth disease virus (FMDV) belongs to the *Picornaviridae* family. FMD viruses are classified in one of seven serotypes (A, O, C, Asia, South African Territories [SAT] serotype 1, SAT2, and SAT3) and show remarkable intra-serotype variability. FMD viruses are highly contagious and outbreaks have great economic burden. Consequently epidemiological monitoring is very important but it is baffled by continues antigenic variation of capsid proteins.
In vivo, the host immune response is a selective constriction operating during virus replication and virus can evade it trough genetic variation. Escape from cytotoxic T cells or from neutralizing antibodies may contribute to viral persistence and disease progression (Narayan *et al.*, 1981; Ciurea *et al.*, 2000). Viral mutants escape monoclonal antibody neutralization commonly by substituting amino acids at the relevant epitope, thus decreasing the affinity with the antibody (Klasse *et al.*, 2002; Mateu *et al.*, 1995).
Therefore, studies of FMDV antigenic sites are important and may contribute to a better control of the disease. They can be done mainly using monoclonal antibodies or viral peptides. The latter methods allow to study sequential epitopes only, while the former combined with mapping of mutations allow the identification of any kind of antigenic site (either linear or conformational) involved in neutralization.
Using the monoclonal antibodies approach, several antigenic sites have been identified and mapped on FMDV type O (Pfaff *et al.*, 1988; Barnett *et al.*, 1989; Kitson *et al.*, 1990; Crowther *et al.*, 1993a; Xie *et al.*, 1987), type A (Thomas *et al.*, 1988; Baxt *et al.*, 1989; Bolwell *et al*, 1989), type C (Mateu *et al.*, 1990; Lea *et al.*, 1994), and type Asia 1 (Grazioli *et al.*, 2004): these studies provided evidence of an underlying structural similarity between the antigenic sites detected in different FMDV serotypes.
The mature FMDV capsid comprises 60 copies of a protomer in turn formed by four proteins: VP1, VP2, VP3, and VP4. The first three proteins present an eight-stranded β-barrel structure in which CHEF strands are exposed on the capsid surface. Their connecting loops show high antigenicity because of their less structural constraints. In particular, the G-H loop in VP1 is a well-described antigenic and immunogenic site found in all serotypes analysed so far. It is usually denominated site I (or site A in serotype C) and is composed by several overlapping epitopes, flanking both sides

of the conserved RGD tripeptide (Stave *et al*., 1988; Bolwell *et al.,* 1989; Mateu *et al.,* 1990), which is critical for an integrin-receptor binding (Jackson *et al.*, 2003 and references therein). A separate site, functionally independent but physically overlapping with site I (critical residue 149) has been reported in virus type O (Crowther *et al.*, 1993a): it was supposed to originate from interaction of the VP1 loop region with other surface amino acids. The highly exposed C-terminus of VP1, particularly the sequence 200-213, has also been implicated in antigenicity of site I, either in combination with residues in the G-H loop (Xie *et al* 1987, Baxt *et al.,* 1989, Parry *et al.,* 1989) or as a topologically independent linear site (Thomas *et al.,* 1988, Mateu *et al.*, 1990). Moreover, other epitopes involved in virus neutralisation were found across different serotypes (Mateu, 1995; Grazioli *et al.*, 2004). Site II involves several amino acids in the B-C loop of VP2, spanning from positions 67 to 79. Site III, only found in type O virus, was placed inside the B-C loop of VP1 (critical positions were residues 43-45 and 48, Barnett *et al.*, 1989, Kitson *et al.*, 1990). Site IV maps within the β-B knob of VP3, with crucial amino acids at positions 58-59. Site V, recently described in type Asia 1 virus, is located within the VP3 C-terminus, with an antibody-binding residue at position 218 (Grazioli *et al.*, 2004). All the sites were ascertained as conformational with the exception of site I.
Very little is known about the antigenic structure of the SAT serotypes. Because of the endemic presence of SAT strains in Africa and the market globalization, it is fundamental to learn more about antigenic determinants of these strains to maintain updated knowledge and epidemic-control tools.
Then, our objective was the study of antigenic sites in both SAT1 and SAT2 strains by means of serotype-specific monoclonal antibodies neutralising virus infectivity.

Materials and Methods:

Virus strains. SAT1 Botswana (BOT) 1/68 and SAT2 Zimbabwe (ZIM) 5/81 were obtained from the FAO World Reference Laboratory, Pirbright, UK. They were propagated in swine kidney cells (IBRS-2) and culture supernatants were harvested when maximum cytopathic effect was observed.

Monoclonal antibodies (MAbs) production. Viruses were inactivated with binary ethyleneimine treatment, precipitated with 8% PEG-6000 and purified by ultracentrifugation through a 25% (w/w) sucrose cushion to be used as antigens. Balb/c mice were initially primed with 50µg of viral antigen with Freud's adjuvant and subsequently boosted with 50µg of antigen alone. Mice were sacrificed after they showed an adequate antibody production; three days after the final booster spleen cells were recovered and fused with NSO cells in presence of PEG-4000. Hybridoma were selected using medium supplemented with HAT, MAbs production was screened by trapping ELISA against homologous virus and positive cultures were carried on (Galfré *et al.*, 1981; Brocchi *et al.*, 1993). Hybridoma culture supernatants or ascitic fluids harvested after *in vivo* culture of hybridoma were used as source of MAbs.

Virus Neutralization test (VNT). To test MAbs neutralization capacity, 100 $TCID_{50}$ of virus was added to MAbs serial dilutions in 96 wells plates, followed by IBRS-2 cells. Cytopathic effect was assessed and dilution required to neutralize 50% of inoculated cultures was determined for each MAb (Brocchi *et al.*, 1995).

Immunoblot test, Hybridoma supernatants were assayed against the purified homologous FMD virus resolved by 12% SDS-PAGE and transferred onto nitrocellulose filters following standard procedures (Harlow & Lane, 1988).

Selection of MAb Neutralization-Resistant mutants (MAR-mutants). Briefly, mutants were selected by mixing in microplate wells appropriate dilutions of MAb (ascitic fluid 1/50 or hybridoma culture supernatants 1/20) with serial tenfold dilutions of the parental FMD virus and by adding subsequently IBRS-2 cells. Supernatants of wells showing cytopathic effect were sub-cultured with increasing concentration of MAb (up to 5X) for two-three passages and then expanded without MAb for further analyses (Borrego *et al.,* 2002).

Trapping ELISA. A trapping ELISA test was used for the screening of hybridoma, for MAbs titration and to characterise MAR-mutants. ELISA microplates were coated with saturating concentrations of rabbit anti-SAT1 or anti-SAT2 antibodies. Appropriate dilutions of parental or mutant strains (supernatant of infected cells) were trapped, MAbs were successively incubated (in serial dilutions to determine antibody concentration or at a saturating dilution when evaluating their reactivity with escape mutants) and the positive reaction was revealed by addition of anti-mouse immunoglobulins, peroxidase–labelled. OPD with H_2O_2 was used to develop colour, reaction was

stopped with 2N H_2SO_4 and optical density values read at 490nm. Reactivity of mutants with each MAb was expressed as a percentage of the corresponding reaction with the parental strain, assumed 100%, while MAbs titres were expressed as the highest saturating dilution. A similar trapping ELISA with type-specific catching sera and FMDV strains representative of each serotype (as indicated in table 1) was used to evaluate the cross-reactivity of MAbs against heterologous FMDV serotypes.

PCR and Sequencing. RNA was extracted from supernatant of infected cultures using a commercial kit (QIAgen RNeasy Mini kit), reverse transcription was performed (RT-PCR) using AMV reverse transcriptase and random primers (Roche); the resulted cDNA was used to amplify the polyprotein P1. Separated reactions were carried out with three distinct sets of type-specific primers (Table 1): VP1, VP2, VP3, VP4 coding regions, and some flanking region both upstream and downstream P1 were amplified. Sequencing of PCR fragments and sequence analysis were carried out using ABI 310 Automatic Sequencer and LaserGene software.

Table 1. Primers used for amplification and sequencing of the capsid coding region (poliprotein P1)

	Primer	Position	Sequenze	Product Size
FMDV type SAT 1	*1618D*	1618-1635	5'- AGGATCGCGCTCGTCTGT -3'	796 bp
	2414R	2414-2391	5'- AGTCCCCTGGTCGTGCCTGTCAAC -3'	
	2318D	2318-23-41	5'- CACCCTTTTCCCACACCAGTTCTT -3'	760 bp
	3078R	3078-3055	5'- CGTCAGCGTAGGTGTAGGCGTAAT -3'	
	3012D	3012-3031	5'- AACTCGACTTTCACCTTCAC -3'	906 bp
	3918R	3918-3896	5'- ACTCAACGTCTCCAGCCAACTTT -3'	
FMDV type SAT 2	*401D*	401-422	5'- ATTTCCACGCCGGCATTTTCAT -3'	968 bp
	1369R	1369-1347	5'- GCTTGCCCTGATCGTGCCTGTTC -3'	
	1243D	1243-1265	5'- TCGCTCAAGGACAGAGAGGAGTT -3'	787 bp
	2030R	2030-2010	5'- GTGTGTGTGTAGGAGAAGTCAA -3'	
	1932D	1932-1950	5'- AGCCCACTGCTACCACTCG -3'	962 bp
	2894R	2894-2873	5'- CTGACGTCGGAGAAGAAGAAGG -3'	

FMDV SAT1 BOT iso47 (Accession Nr. AY593845) sequence was used to design the SAT1-specific primers: positions indicated refer to that sequence. FMDV SAT2 ZIM/7/83 (Accession Nr. AF540910) sequence was used to design SAT2 primers: we changed annotation and the position 1 corresponds to nucleotide in position 1058 of the original sequence.

Results:

Two panels of monoclonal antibodies were obtained, composed by 29 and 12 MAbs raised against FMDV type SAT 1 (strain Botswana 1/68) and SAT 2 (strain Zimbabwe 5/81) respectively. An overview of their characteristics is reported in table 2.
The most frequent isotype in both panels is IgG1.
All MAbs reacted strongly in trapping ELISA with the homologous virus strain captured by immune rabbit sera, with titres (expressed as highest saturating dilution) in hybridoma supernatants ranging from 1/5 to 1/625. The reaction was type-specific, with the exception of two MAbs of the SAT1 panel, of which one cross-reacted with FMDV type Asia 1 and another recognised the seven FMDV serotypes to various extent, the three SAT types more intensely.
Twenty-two anti-SAT 1 MAbs and five anti-SAT 2 MAbs neutralized infectivity of the homologous virus with variable titres. Of these, nine anti-SAT 1 and three anti-SAT 2 MAbs reacted with homologous denatured VP1 in immunoblot tests, substantiating that their target sites lay in a linear amino acid sequence. Two further MAbs against the SAT 1 type showed a profile of reactivity in immunoblot distinguishable from the previous ones, recognizing a band of slightly lower molecular weight: the position of amino acid substitutions found in the corresponding MAR-mutants (see following paragraph) suggests the target protein detected in immunoblot is most likely VP3.
The other neutralizing MAbs, that did not recognize any denatured viral protein in immunoblot, are likely directed to conformation-dependent sites, similarly to 13 out of the 14 non-neutralizing MAbs, distributed seven in each panel.

While the non-neutralizing MAbs were not further studied, the antigenic sites target of the neutralizing MAbs were identified and mapped on the capsid proteins. The approach adopted to this purpose was the selection of viral mutants resistant to MAb neutralization and the subsequent detection of amino acid substitutions responsible for mutation.

Selection and characterisation of MAb-resistant mutants (MAR-mutants)

FMDV type SAT 1
For each of 8 strongly neutralizing MAbs, 6 to 23 MAR-mutants were selected from independent events. The antigenic profile of each mutant was then determined by analyzing its reactivity with all the neutralizing MAbs in a trapping ELISA (figure 1). Independent mutants obtained with the same MAb showed identical or very similar patterns of reactivity; MAbs that lost reactivity with the same mutants were assigned to the same group. The whole pattern allowed the identification of five distinguishable profiles, indicative of five distinct neutralization sites. The independence between sites is suggested by evidence that antigenic mutation occurred in one site did not affect the reactivity of the other sites.
In particular, one site is defined by the single MAb 4B9, another site by the eight MAbs 4C5, 4G12, 3C9, 3A3, 4C4, 4F3, 3G4, 3F9 and a third site is identified by the five further MAbs 4B1, 3E5, 4E3, 3F2, 4F2. Some variation observed in the profile of the mutants within this third site is suggestive of a more complex determinant, probably composed by heterogeneous yet related epitopes. Two additional distinct sites are defined by clusters of four (3E4, 4D4, 3C10, 3B6) and two (4H8, 2D2) MAbs respectively.

FMDV type SAT 2
Only three MAbs specific to the SAT 2 type (2H6, 4A6, 3C5), known to recognize linear epitopes in VP1 according with results of immunoblot tests, had neutralizing titres that allowed the selection of resistant mutants.
Seventeen 2H6-resistant mutants and ten 4A6-resistant mutants were isolated; all of them showed identical patterns of reactivity in the profiling ELISA, proving both MAbs have a common target site, that is structurally independent from other MAbs binding sites (figure 2).
MAb 3C5 identified a different neutralizing epitope, as the seventeen 3C5-resistant mutants selected maintained full reactivity with all MAbs except 3C5.

Mapping of neutralizing sites by sequencing of MAR-mutants
The capsid-coding region of the parental viruses (FMDV SAT1 Botswana 1/68 and FMDV SAT2 Zimbabwe 5/81) and some MAR-mutants were sequenced and the deduced amino acid sequences were compared to identify changes responsible for site mutation. General observations were: (i) mutants selected with MAbs defining one site showed amino acid changes at the same or contiguous residues, but there were also instances of two simultaneous changes in residues located in different structural proteins; (ii) in most cases different amino acids could replace the same position causing analogous effects.

FMDV type SAT 1 mutants (figure 1, details in table 3)
According with results of mutants sequencing, that confirmed the previous classification of distinct antigenic sites, the neutralizing sites identified in the SAT 1 capsid structure were named I (a and b) VI, VII, VIII in order to maintain and continue the denomination already given to sites I to V, previously described in other FMDV serotypes.

Site I was located in VP1 and appeared to be composed by distinguishable epitopes; mutants selected with MAb 4B9 (site Ia) showed each a unique substitution at residue 154, or 156, or 157 of VP1; mutants selected with MAb 4C5, representative of a group of 8 MAbs with a similar profile (site Ib), showed substitutions at residue 146 of VP1, either alone or combined with a simultaneous substitution at the contiguous residue 148. The positions defining site Ia and Ib flank both sides of the conserved RGD motif and are included in the flexible GH loop of VP1, protruding from the capsid surface: we considered epitopes Ia and Ib, in spite of their reciprocal independence, distinguishable portions of the main antigenic site I, that was also described in all other FMDV types characterized so far.

Site VI was considered a novel domain since it involves amino acid residues not found in other FMDV sites. Furthermore, sequencing of mutants selected with MAbs of site VI confirmed its heterogeneity. The three 4B1-mutants sequenced showed a unique amino acid change at residue 135 of VP3. This change alone was enough to knock down the reactivity in ELISA of all MAbs belonging to site VI group.

The mutation in position 135 of VP3 was found also in some 3E5-, 4E3- and 3F2-derived MAR mutants. In all these mutants but one, it was associated with an additional change in either position 179 or 181 of VP1. Other 3E5, 4E3, and 3F2 mutants showed the latter mutation in association with a change at residue 71 or 76 of VP3.
Considering all the MAR-mutants in this group, site VI seems to include an epitope mapping at the dominant position VP3 135 and further related epitopes that involve two residues simultaneously, one in VP3 (positions 135 or 71 or 76) and one in VP1 (positions 179 or 181). In order to envisage structural relationship between residues involved in site VI, the relevant positions were projected onto a 3D model of the protomer structure constructed using the sequence of the parental virus, FMDV type SAT 1, strain Botswana 1/68.
Residues 71, 76 and 135 resulted clustered near one another on the outward surface of VP3 and close to the icosahedral threefold axis. Residues 179 and 181 in VP1 were also spatially near to each other but structurally distant from VP3 residues and almost hidden from outer viewpoint (not shown). Whether changes in VP1 are a consequence to compensate changes in VP3 or they are an effective part of the antigenic site is uncertain. It is interesting to note that MAbs 4E3 and 3F2 of this group, that according with their reactivity profile and common changes induced in the generated mutants should identify an identical epitope, recognized in immunoblot a protein slightly smaller than VP1, then presumably VP3. This suggests that at least a portion of the epitope should contain a linear sequence located in VP3, sufficient to be recognized by these MAbs.
Another remarkable observation regards the epitope target of MAb 4B1, in that it was the only one to vanish in the viral sub-particle 12S (obtained by heat treatment, results not shown), trapped by either policlonal or monoclonal antibodies in a trapping ELISA. This behaviour is consistent with results of epitope mapping, that assigned a crucial role to residue VP3 135, located close to the icosaehedral threefold axis, and then sensible to the degradation of 146S particles to 12S sub-particles.

Based on changes repeatedly detected in three MAR-mutants selected with MAb 3E4, **site VII** was found to involve two amino acids in two different proteins, namely positions 181 of VP1 and 72 of VP2. In the 3D model of the protomer structure, these positions appeared distant one another and unrelated. Moreover the two residues are separately involved in the structure of other neutralizing sites: in fact we showed that position VP1 181 is related to site VI in type SAT 1, while it has been reported that position VP2 72 is included in site II described in various FMDV serotypes (O, A, C and Asia 1).

Finally, **site VIII** was mapped at position 111 of VP1, that was found substituted in all of five MAR-mutants sequenced. Further simultaneous changes were detected in a couple of mutants, involving residues lying in the GH loop of VP1 (site Ib); consistently, one of these mutants lost reactivity also with MAbs to site Ib. Then, a double mutation affecting site I and site VIII should have occurred in these mutants.

FMDV type SAT 2 mutants (figure 2, details in table 4)
The profile of reactivity of MAR-mutants and results of immunoblot tests showed that MAbs specific for the SAT 2 type identify two distinguishable linear sites in VP1. Accordingly, the sequence of representative mutants provided evidence that the site target of MAbs 2H6 and 4E6 is located within the C-terminus of VP1 and consistently involves the amino acid position 210, while the site defined by MAb 3C5 incorporates residue 154 of VP1, alone or more frequently combined with changes in amino acid VP2 79. Residue VP1 154 is included within the GH loop of VP1 so that the antibody-contact region should correspond to the well known site I, whilst the function of residue VP2 79 in this site remains obscure.

Discussion:

Purpose of this work was to investigate the antigenic structure of FMDV serotypes SAT 1 and SAT 2 by means of MAbs to identify antigenic sites involved in neutralization of virus infectivity.
A number of papers reported on the identification and mapping of neutralizing sites in serotypes O, A, C and Asia 1: antigenic sites showed great similarity between serotypes (Mateu *et al.,* 1995, Grazioli *et al*, 2004). However, little is known about the SATs serotypes; to date, only MAbs specific to a type SAT 2 FMD virus have been described and all epitopes identified were associated with the VP1 G-H loop region (Crowther *et al.*, 1993b).
The characterization of new panels of MAbs specific to SAT 1 and SAT 2 serotypes combined with the location of amino acids substitutions conferring resistance to neutralizing MAbs, led to two major findings: confirmation of the presence of a major immuno-dominant site at G-H loop of both SAT 1 and SAT 2 VP1, and the detection of three novel conformational sites in the SAT 1 structure, that were not found in other previously studied serotypes.

In the SAT 1 virus type the antigenic site I was defined by two sub-sets of MAbs, whose critical antibody-contact residues were located upstream (residue 146, 148) and downstream (residues 154, 156, 157) the RGD tripeptide. In FMDV this conserved motif mediates the attachment to the cell receptor (Jackson *et al.*, 2003 and references therein) and, in contrast to the receptor-attachment sites in other picornaviruses, it is located at the tip of the most exposed loop and is accessible to antibodies (Verdaguer *et al*., 1995). Consistently with previous findings in other FMDV serotypes, it appears that variation in the sequences at either side of RGD serves to preserve these vital residues from antibody binding, retaining the capability of virus to infect susceptible cells by binding to the cellular receptor (Acharya *et al*., 1989).
In the SAT 2 serotype both structural elements described to compose site I, namely VP1 G-H loop and C-terminus, have been identified with our MAbs panel.
The antibody-contact point in the G-H loop (MAb 3C5) was found to include residue 154, in agreement with results of immunoblot, which assigned the epitope target of MAb 3C5 to a linear sequence of VP1. However, a simultaneous substitution found in several MAR-mutants of the same group at position 79 of VP2 suggests an interaction between site I and other surface-exposed sites, given that VP2 79 is one of the several residues within the VP2 B-C loop associated with site II.
The antibody-contact residue in the VP1 C-terminus was found to be at position 210. In other FMDV serotypes, VP1 C-terminus was described as part of a discontinuous site together with site I (Xie *et al* 1987, Baxt *et al.,* 1989, Parry *et al.,* 1989), or as an independent site of minor importance, due to the weak neutralizing capacity of MAbs towards it and their failure to compete with convalescent sera (Thomas *et al.,* 1988, Mateu *et al.,* 1990). Our data provided evidence that in the SAT 2 virus the VP1 C-terminus contains an independent and immuno-dominant linear domain, as proven by the proportion of MAbs elicited, their strong neutralizing activity, the reactivity in immunoblot with denatured VP1 and the distinct profile of reactivity with groups of MAR-mutants.

The variety of the anti-SAT 1 MAbs panel allowed the identification of three novel antigenic sites involved in virus neutralization, never described before and for this reason called site VI, VII, VIII.
Site VI is an heterogeneous, structurally complex site, having a key position at the surface-exposed residue 135 of VP3. Changes of this amino acid generated mutants (selected by MAb 4B1) that became resistant to neutralization by all MAbs (data not shown) of the panel described, irrespective of the target site and despite their conserved binding in ELISA. The vicinity of residue VP3 135 to the threefold axis of symmetry, corresponding to conjunction-points between pentamers, may explain the observed disruption of this epitope in the pentameric 12S sub-particles. However, other epitopes within site VI involving two distant residues simultaneously, of which one in VP3 and another in VP1, were conserved in the 12S particles. Residues of VP3, namely position 135 again or 72 or 76, forms a cluster on the outer surface in contrast with residues in VP1, at positions 179 or 181, that appears to be internalized. The recognition in immunoblot of a denatured protein likely corresponding to VP3 by some MAbs defining this site should suggest that antibody-binding residues are those located in VP3, while substitutions in VP1 could be due to structural rearrangements following a distance effect.
Site VII is another peculiar site involving residues at positions VP1 181 and VP2 72. It could be related to the antigenic site II previously described in four serotypes; in fact, only residue VP2 72 is surface-exposed and is part of the antigenic site II. Accordingly, position VP2 72 is more likely to be an antibody-binding residue, while change in VP1 181 could derive from consequent rearrangement. However, the opposite cannot be ruled out, as well as the possibility that both residues are involved in the MAb-binding region.
Finally, the position 111 of VP1 defined an additional novel antigenic site in FMDV serotype SAT 1, thus called site VIII.
In conclusion, MAbs confirmed their power to discover the antigenic structure of FMD viruses. In serotypes SAT 1 and 2 immuno-dominant sites have been confirmed within the G-H loop and C-terminus of VP1, but also three structurally and topologically new sites were discovered studying the SAT 1 serotype: they could represent new major determinants in FMD viruses, or may denote a different antigenic structure in SAT serotypes.
These data may have useful application in diagnostic and epidemiological investigations, as MAbs directed against conserved epitopes provide universal reagents for FMDV detection systems, while MAbs against known variable sites readily allow the identification of antigenic variants. In preliminary studies, simple sandwich ELISAs performed using these MAbs as coating and conjugated antibody were proven to reliably detect and type the specific antigens. Studies now in progress are directed to validate diagnostic performances of such assays, by extending evaluation on clinical samples, and to analyse the antigenic variability of the target sites, by examining the MAbs reactivity against different isolates.

Conclusions:

- A further step was done in characterization of the antigenic structure of FMD viruses by using monoclonal antibodies.
- The G-H loop and C-terminus of VP1 were confirmed to contain a major antigenic determinant also in SAT serotypes, and in addition three novel conformational sites were detected in SAT 1 serotype. The latter could represent new major determinants in FMD viruses, or denote significant differences between antigenic structure of SAT and other serotypes.
- MAbs are valuable diagnostic tools for the development of antigen and antibodies typing.

Recommendations:

- Characterization of MAbs and of the relevant target sites is of primary importance in order to select appropriate panels for different applications, such as diagnostic tests or antigen profiling, and to guide to a correct interpretation of results.
- Since immunoassays benefit from the use of MAbs, the development of MAbs-based assays and their stabilization towards kit format should be encouraged.

Acknowledgments:

We thank Dr. D. Gamba for hybridoma cultures and Dr. M. Bugnetti for virus cultures. Work was partially supported by Italian Ministry of Health (grant IZSLER 15/03) and by EU (grant SSPE-CT2003-513645 LabonSite and 503603 FMD_ImproCon).

References:

Acharya, R., Fry, E., Stuart, D., Fox, G., Rowlands, D. & Brown, F. 1989. The three-dimensional structure of foot-and-mouth disease virus at 2.9 A resolution. *Nature*, 337: 709-16.

Barnett, P.V., Ouldridge, E.J., Rowlands, D.J., Brown, F. & Parry, N.R. 1989. Neutralizing epitopes on type O foot-and-mouth disease virus. I. Identification and characterization of three functionally independent, conformational sites. *Journal of General Virology*, 70: 1483-1491.

Baxt, B., Vakharia, V., Moore, D. M., Franke, A.J. & Morgan, D.O. 1989. Analysis of neutralizing antigenic sites on the surface of type A_{12} foot-and-mouth disease virus. *Journal of Virology*, 63: 2143-2151.

Bolwell, C., Clarke, B.E., Parry, N.R., Ouldridge, E.J., Brown, F. & Rowlands, D.J. 1989. Epitope mapping of foot-and-mouth disease virus with neutralizing monoclonal antibodies. *Journal of General Virology*. 70: 59-68.

Borrego, B., Carra E., Garcia-Ranea, J.A. & Brocchi, E. 2002. Characterization of neutralization sites on the circulating variant of swine vesicular disease virus (SVDV): a new site is shared by SVDS and the related coxsackie B5 virus. *Journal of General Virology*, 83: 35-44.

Brocchi, E., Gamba, D., Poumarat, F., Martel, J. L. & De Simone, F. 1993. Improvements in the diagnosis of contagious bovine pleuropneumonia through the use of monoclonal antibodies. *Revue Scientifique et Technique Office International des Epizooties*, 12: 559-570.

Brocchi, E., Berlinzani, A., Gamba, D. & De Simone, F. 1995. Development of two novel monoclonal antibody-based ELISAs for the detection of antibodies and the identification of swine isotypes against swine vesicular disease virus. *Journal of Virological Methods*, 52: 155-167.

Ciurea, A., Klenerman, P., Hunziker, L., Horvath, E., Senn, B.M., Ochsenbein, A.F., Hengartner, H. & Zinkernagel, R.M. 2000**.** Viral persistence in vivo through selection of neutralizing antibody-escape variants.
Proceedings of the National Academy of Science of The United States of America, 97: 2749-2754.

Crowther, J.R., Farias, S., Carpenter, W.C. & Samuel, A.R. 1993a. Identification of the fifth neutralizable site on type O foot-and-mouth disease virus following characterization of a single and quintuple monoclonal antibody escape mutants. *Journal of Genetic Virology*, 74: 1533-1547.

Crowther, J.R., Rowe, C.A. & Butcher, R. 1993b. Characterization of monoclonal antibodies against a type SAT 2 foot-and-mouth disease virus. *Epidemiology and Infection*, 111: 391-406.

Galfré, G. & Milstein, C. 1981. Preparation of monoclonal antibodies: strategies and procedures. *Methods in Enzymology*, 73: 3-46.

Grazioli, S., Fallacara, F., & Brocchi, E. 2004. Mapping of neutralising sites on FMD virus type Asia 1 and relationships with sites described in other serotypes. Report of Sess. Research Group St. Tech. Committee of the European Commission for the Control of Foot-and-Mouth Disease, Crete – 11-15 October 2004 –Appendix 44, p 277-287.

Harlow, E. & Lane, D. 1988. Immunoblotting protocols. In: Antibodies: a Laboratory manual, p 479-510. Cold Spring Harbor, NY: Cold Sring Harbor laboratory.

Jackson, T., King, A.M.Q., Stuart, D.I., & Fry, E. 2003. Structure and receptor binding. *Virus research*, 91:33-46.

Kiston, J.D.A., McCahon, D. & Belsham, G.J. 1990. Sequence analysis of monoclonal antibody mutants of type O foot-and-mouth disease virus: evidence for involvement of the three surface exposed capsid protein in four antigenic sites. *Virology*, 179: 26-34.

Klasse, P.J. & Sattentau, Q.J. 2002**.** Occupancy and mechanism in antibody-mediated neutralization of animal viruses. *Journal of General Virology*, 83: 2091-2108.

Lea, S., Hernàndez, J., Blakemore, W., Brocchi, E., Curry, S., Domingo, E., Fry, E., Abu-Ghazaleh, R., King, A., Newman, J., Stuart, D. & Mateu, M.G. 1994. The structure and antigenicity of a type C foot-and-mouth disease virus. *Structure*, 2: 123-139.

Mateu, M.G., Martinez, M.A., Capucci, L., Andreu, D., Giralt, E., Sobrino, F., Brocchi, E. & Domingo, E. 1990. A single amino acid substitution affects multiple overlapping epitopes in the major antigenic site of foot-and-mouth disease virus of serotype C. *Journal of General Virology*, 71: 629-637.

Mateu, M.G. 1995**.** Antibody recognition of picornaviruses and escape from neutralization: a structural view. *Virus Research*, 38: 1-24.

Narayan, O., Clements, J., Griffin, D.E. & Wolinsky, J.S. 1981. Neutralizing antibody spectrum determines the antigenic profiles of emerging mutants visna virus. *Infection and Immunity*, 32: 1045-1050.

Parry, N.R., Barnett, P.V., Ouldridge, E.J., Rowlands, D.J. & Brown, F. 1989. Neutralizing epitopes of type O foot-and-mouth disease virus. II. Mapping three conformational sites with synthetic peptide reagents. *Journal of General Virology*, 70:1493-1503.

Pfaff, E., Thiel, H.J., Beck, E., Strohmaier, K. & Schaller, H. 1988. Analysis of neutralizing epitope on foot-and-mouth disease virus. *Journal of Virology*, 62: 2033-2040.

Stave, J.W., Card, J.L., Morgan, D.O. & Vakharia, V.N. 1988. Neutralization sites of type O1 foot-and-mouth disease virus defined by monoclonal antibodies and neutralization-escape virus variants. *Virology*, 162: 21-29.

Thomas, A.A.M., Woortmeijer, R.J., Puijk, W. & Barteling, S.J. 1988. Antigenic sites on foot-and-mouth disease virus type A10. *Journal of Virology*, 62**,** 2782-2789.

Verdaguer, N., Mateu, M.G., Andreu, D., Giralt, E., Domingo E. & Fita, I. 1995. Structure of the major antigenic loop of foot-and-mouth disease virus complexed with a neutralizing antibody: durect involvement of the Arg-Gly-Asp motif in the interaction. *EMBO Journal*, 14: 1690-6.

Xie, Q.C., McCahon D., Crowther, J.R., Belsham, G.J. & McCullough, K.C. 1987. Neutralization of foot-and-mouth disease virus can be mediated through any of at least three separate antigenic sites. *Journal of General Virology*, 68: 1637-47.

Table 2. Reactivity of MAbs raised against FMDV type SAT 1 and SAT 2

	Ig class	Mab code	VNT titre (homologous) Hybridoma supernatant	VNT titre (homologous) ascitic fluid	Western Blot (hologous virus)	ELISA Trapping: **SAT 1** Bot 1/68 homolog. (titre)	**SAT 2** Zim 5/81 homologous (titre)	**SAT 3** Zim 4/81	**O** UK 2001	**A 22** Iraq	**Asia 1** Nep29/97	**C1** Italy 64	Ag site mapping
MAbs raised against FMDV type Sat 1, strain Bot 1/68													
Neutralising	nd	**4B9**	1/128	≥20484	VP1 w	1/125	-	-	-	-	+	-	VP1 154-157
	G1/G2b	**4C5**	1/768	1/20484	VP1	1/625	-	-	-	-	-	-	VP1 146 & 148
	G1	**4G12**	1/128	na	VP1	1/625	-	-	-	-	-	-	
	G1	**3C9**	1/128	na	VP1	1/625	-	-	-	-	-	-	
	G1	**3A3**	1/128	na	VP1	1/125	-	-	-	-	-	-	
	G1	**4C4**	1/64	na	VP1 w	1/25	-	-	-	-	-	-	
	G1/G2a	**4F3**	1/32	na	VP1 w	1/5	-	-	-	-	-	-	
	G1	**3G4**	1/32	na	VP1 w	1/5	-	-	-	-	-	-	
	G1	**3F9**	1/16	na	VP1 w	1/5	-	-	-	-	-	-	
	G2a	**4B1**	>1/256	≥20484	-	1/125	-	-	-	-	-	-	VP3 135
	G1	**3E5**	>1/128	nd	±	1/625	-	-	-	-	-	-	VP3 135 & VP1 179-181 & VP3 71-76
	G1	**4E3**	1/12	1/5120	VP3 ?±	1/125	-	-	-	-	-	-	
	G2b	**3F2**	>1/128	nd	VP3 ?	1/625	-	-	-	-	-	-	
	G1	**4F2**	1/8	na	+ w	1/25	-	-	-	-	-	-	
	G2b	**3E4**	1/16	1/9720	-	1/5	-	-	-	-	-	-	VP1 181 & VP2 72
	G1/M	**4D4**	>1/128	na	-	1/625	-	-	-	-	-	-	
	G1/M/A	**3C10**	1/8	na	-	1/25	-	-	-	-	-	-	
	G2a	**3B6**	1/16	na	-	1/5	-	-	-	-	-	-	
	G1	**4H8**	>1/128	1/9720	-	1/625	-	-	-	-	-	-	VP1 111
	G1	**2D2**	1/16	nd	-	1/25	-	-	-	-	-	-	
	G1/G2b	**4F11**	1/16	na	-	1/125	-	-	-	-	-	-	not mapped
	G1/M	**3E3**	1/4	na	-	<1/5	-	-	-	-	-	-	
Non-neutralising	G1	**4D3**	-	na	-	1/625	-	-	-	-	-	-	unknown
	G1/G2b	**4A6**	-	na	-	1/625	-	-	-	-	-	-	
	G1	**3F3**	-	na	-	1/125	-	-	-	-	-	-	
	G1	**4H10**	-	na	-	1/25	-	-	-	-	-	-	
	G1/M	**4G5**	-	na	-	1/5	-	-	-	-	-	-	
	M	**3D1**	-	na	-	1/5	-	-	-	-	-	-	
	G1	**4E8**	-	na	-	1/5	+	+	±	±	+	±	
MAbs raised against FMDV type Sat 2, strain Zim 5/81													
Neutralising	G1	**3C5**	1/1536	1/20480	+ VP1	-	>1/625	-	-	-	-	-	VP1 154
	G1/M	**4A6**	1/32	>1/20480	+ VP1	-	1/25	-	-	-	-	-	VP1 210
	G1	**2H6**	1/16	1/15360	+ VP1	-	1/25	-	-	-	-	-	VP1 210
	G1	**2A8**	1/6	1/30	**-**	-	1/5	-	-	-	-	-	not mapped
	G1/M	**4C7**	1/2	na	-	-	1/625	-	-	-	-	-	
Non-neutralising	G1	**1F5**	-	<10	**-**	-	1/25	-	-	-	-	-	unknown
	G1	**4F4**	-	<10	-	-	1/125	-	-	-	-	-	
	G1/M	**2F4**	-	<10	-	-	1/25	-	-	-	-	-	
	G1	**4H5**	-	na	**-**	-	1/5	-	-	-	-	-	
	G1	**4A8**	-	na	-	-	1/5	-	-	-	-	-	
	G1	**1F3**	-	na	-	-	1/5	-	-	-	-	-	
	G1/M	**4G6**	-	na	**+ VP?**	-	1/5	-	-	-	-	-	

na = not available w = weak positive
VNT titres = end-point titres ELISA titres = highest saturating dilution

Figure 1. Profile of reactivity in ELISA of FMDV SAT 1 mutants with the panel of neutralising MAbs

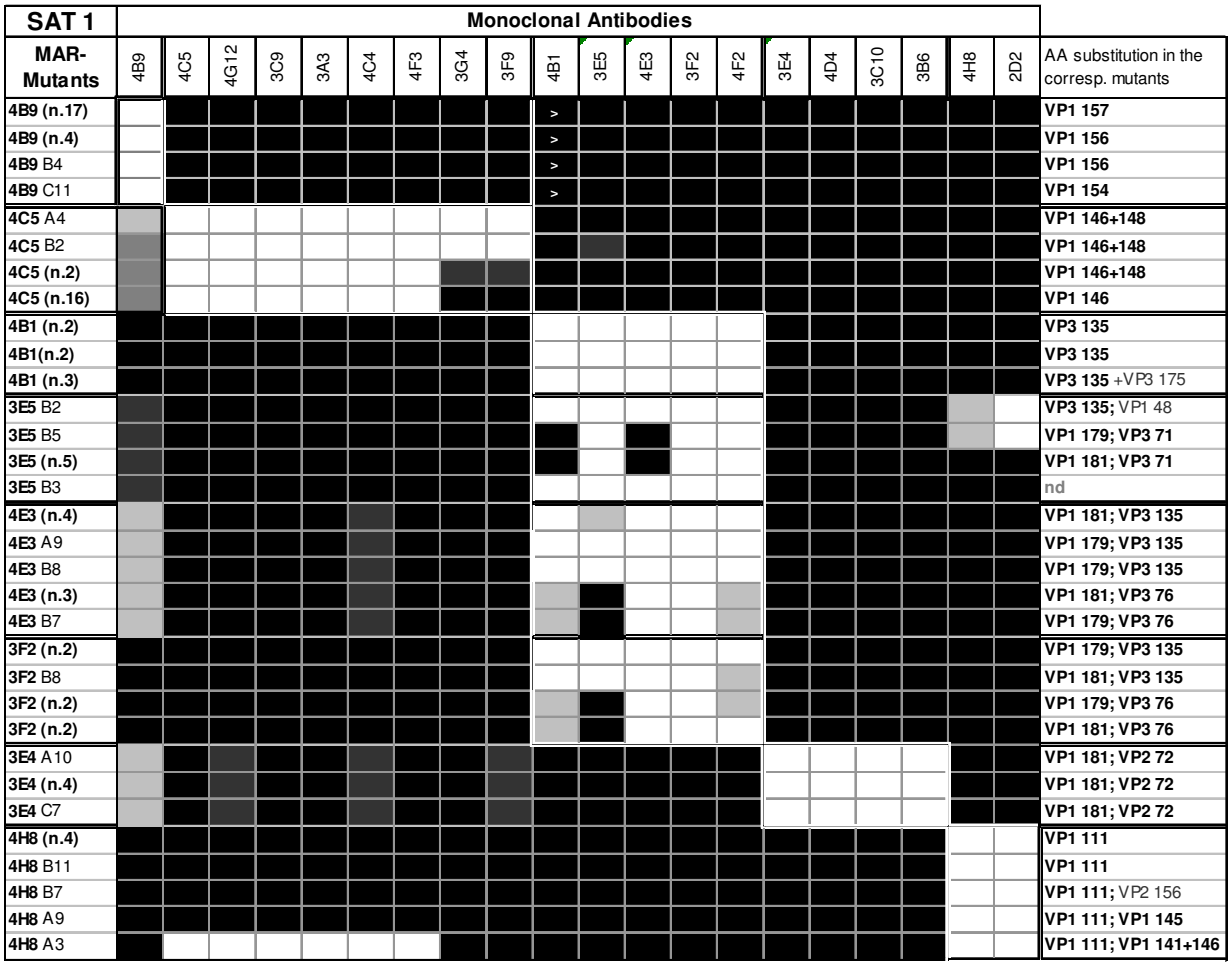

Figure 2. Profile of reactivity in ELISA of FMDV SAT 2 mutants with the panel of neutralising MAbs.

SAT 2	Monoclonal Antibodies					
MAR-Mutants	3C5	4A6	2H6	4C7	2A8	AA substitution in the corresp. mutants
2H6 (n.15)	■			■	■	VP1 210
2H6 A10	■			■	■	VP1 210
2H6 B5	■			■	■	VP1 210
4A6 (n.6)	■			■	■	nd
4A6 (n.4)	■			■	■	VP1 210
3C5 (n.14)		■	■	■	■	VP1 154; VP2 79
3C5 A6		■	■	■	■	VP1 154; VP2 79
3C5 A9		■	■	■	■	VP1 154; VP2 79
3C5 B8		■	■	■	■	VP1 154

	< 30%
	30% → 70%
■	70% →100%
>	>100%

Percentage of reactivity related to the parental virus, assumed to be 100%, is shown as indicated

The number of MAR-mutants analysed, showing the same profile of reactivity, is indicated in brackets; of them only one has been selected for sequencing

Amino acid mutations written in grey were not considered part of the epitope but just random occurrences.

Table 3. Aminoacid substitutions found in FMDV type SAT 1 MAR-mutants

Ag site	MAR-mutant	Amino acid changes
Ia	4B9/B5	VP1 157 *(A → P)*
	4B9/A7	VP1 156 *(A → T)*
	4B9/B4	VP1 156 *(A → V)*
	4B9/C11	VP1 154 *(T→ I)*
Ib	4C5/A4	VP1 146 *(E→ G)* ; VP1 148 *(I → T)*
	4C5/B2	VP1 146 *(E → G);* VP1 148 *(I → T)*
	4C5/C10	VP1 146 *(E → G);* VP1 148 *(I → T)*
	4C5/A11	VP1 146 *(E → A)*
VI	4B1/A2	VP3 135 *(E → V)*
	4B1/A8	VP3 135 *(E → G)* ; VP3 175 *(E → G)*
	4B1/C7	VP3 135 *(E → A)*
	3E5/B2	VP3 135 *(E → A);* VP1 48 *(N → K)*
	3E5/B5	VP3 71 *(S→ L);* VP1 179 *(E→ K)*
	3E5/C4	VP3 71 *(S → R);* VP1 181 *(D → A)*
	4E3/C9	VP3 135 *(E → V)*; VP1 181 *(D → N)*
	4E3/A9	VP3 135 *(E → A);* VP1 179 *(E → A)*
	4E3/B8	VP3 135 *(E → A);* VP1 179 *(E → A)*
	4E3/A11	VP3 76 *(C → R);* VP1 181 *(D → A)*
	4E3/B7	VP3 76 *(C → R);* VP1 179 *(E → A)*
	3F2/B4	VP3 135 *(E → V);* VP1 179 *(E → K)*
	3F2/B8	VP3 135 *(E→ K);* VP1 181 *(D → G)*
	3F2/B2	VP3 76 *(C → R);* VP1 179 *(E → A)*
	3F2/B10	VP3 76 *(C → R);* VP1 181 *(D → N)*
VII	3E4/A10	VP2 72 *(D → N);* VP1 181 *(D → A)*
	3E4/A11	VP2 72 *(D → N);* VP1 181 *(D → G)*
	3E4/C7	VP2 72 *(D → V);* VP1 181 *(D → N)*
VIII	4H8/B3	VP1 111 *(R →G)*
	4H8/B11	VP1 111 *(R → G)*
	4H8/B7	VP1 111 *(R → G)*; VP2 156 *(A → T)*
	4H8/A9	VP1 111 *(R → G)*; VP1 145 *(R → C)*
	4H8/A3	VP1 111 *(R →G)*; VP1 141 *(G→C)*; VP1 146 *(E → G)*

Table 4. Aminoacid substitutions found in FMDV type SAT 2 MAR-mutants

MAR-mutant	Amino acid changes
2H6/A2	VP1 210 *(G → C)*
2H6/A10	VP1 210 *(G → C)*
2H6/B5	VP1 210 *(G → A)*
4A6/A8	VP1 210 *(G → C)*
3C5/A2	VP1 154 *(Y → H);* VP2 72 *(Y→ H)*
3C5/A6	VP1 154 *(Y → H);* VP2 72 *(Y→ H)*
3C5/A9	VP1 154 *(Y → H ;* VP2 72 *(Y→ H);*
3C5/B8	VP1 154 *(Y → H)*