Recombinant integrin αvβ6 as a capture reagent in immunoassays for the diagnosis of FMD
Nigel Ferris*1, Nicola Abrescia2, David Stuart2,3, Terry Jackson1, Alison Burman1, Donald King1 and
David Paton1

1 Pirbright Laboratory, Institute for Animal Health, Ash Road, Woking, Surrey GU24 0NF, UK
2 Division of Structural Biology, Wellcome Trust Centre for Human Genetics, University of Oxford,
Oxford OX3 7BN, UK
3 Oxford Centre for Molecular Sciences, Central Chemistry Laboratory, University of Oxford, South
Parks Road, Oxford, OX1 3QT, UK

Abstract:
It is thought that the integrin αvβ6 is a principal cell receptor for binding wild-type FMDV. The aim
was to exploit this knowledge by evaluating the performance of recombinant integrin αvβ6 as a
capture ligand in a sandwich ELISA for the detection and serotyping of FMDV. Soluble recombinant
αvβ6 protein was produced from Chinese hamster ovary cells transfected with truncated human αv
and β6 genes and used as the capture reagent in indirect sandwich ELISAs in combination with either
guinea pig polyclonal or monoclonal antibodies (mabs) as detectors. These formats were evaluated
for their ability to react with, and characterise the serotype of, suspensions of field epithelia and a
range of cell culture antigens of all seven serotypes of FMDV plus SVDV. Their performance was also
compared with our conventional assay, which uses polyclonal antisera as capture and detecting
antibodies.

The recombinant αvβ6 protein bound all antigens of FMDV irrespective of serotype but did not react
with SVDV. Heterotypic cross-reactivity was evident with some of the test samples using rabbit/guinea pig polyclonal or monoclonal antibodies (mabs) as detectors. These formats were evaluated
for their ability to react with, and characterise the serotype of, suspensions of field epithelia and a
range of cell culture antigens of all seven serotypes of FMDV plus SVDV. Their performance was also
compared with our conventional assay, which uses polyclonal antisera as capture and detecting
antibodies.

The results illustrate that the use of the recombinant protein as a capture reagent in the ELISA in
combination with appropriate mab(s) as the detector has the potential to improve upon conventional
FMD diagnostic assays using rabbit and guinea pig polyclonal antisera. Additionally, the recombinant
integrin could be usefully used in other immunoassays for FMD diagnosis (e.g. for characterizing the
antigenicity of field virus strains and for detection of FMDV antibody) as well as in other FMDV test
procedures such as immunocapture RT-PCR and pen-side chromatographic strip-test devices.

Introduction:
Diagnosis of FMD is dependent upon early clinical recognition of the disease in the field, followed by
confirmation of the presence and type of FMD virus by objective tests that are usually carried out in
specialised laboratories. The preferred specimen is vesicular epithelium and the most commonly used
laboratory diagnostic tests are the antigen detection ELISA combined with virus isolation in cell
culture (Roeder and Le Blanc Smith, 1987; Ferris and Dawson, 1988) and fluorogenic reverse
transcription polymerase chain reaction (RT-PCR; Reid et al., 2002, 2003). The type of ELISA, which
has been found to be most sensitive is an indirect sandwich assay employing type-specific antibodies
of polyclonal and/or monoclonal origin. The assay is rapid and identifies the serotype of FMD virus
that is present. If necessary, the sensitivity of the method can be enhanced by prior amplification of
virus in cell cultures.

Integrin molecules on cell surfaces are receptors for a number of viruses including field isolates of
FMD virus (Jackson et al., 1997). Integrins are heterodimers comprising α and β subunits, each of
which can occur in a number of different forms. Six or seven different varieties of integrin are known
to bind to the conserved RGD amino acid motif found on the VP1 capsid protein of FMD virus.
However, it is thought that the integrin αvβ6 is the principal receptor for binding wild-type FMD virus
(Jackson et al., 2000). This knowledge could be exploited in a number of ways to develop improved
diagnostic tests for FMD. We have examined firstly the utility of a recombinant protein of αvβ6 to
function as a ligand for FMD virus in the antigen detection ELISA and present the results in this
communication.

Material and Methods:
Preparation of recombinant αvβ6. Recombinant αvβ6 was produced from Chinese hamster ovary
(CHO) cells stably transfected with truncated αv and β6 genes of human origin (Weinacker et al.,
1994) to secrete αvβ6 as a soluble protein in serum-free cell culture supernatant fluids. The
supernatant was clarified by filtration and stored at -20°C.
Polyclonal antibodies. Polyclonal antisera to FMD virus types O, BFS 1860, A<sub>22</sub>/24/26 (Combination), C<sub>3</sub>, Resende, SAT 1 BOT 1/68, SAT 2 ZIM 5/81, SAT 3 ZIM 4/81 and Asia 1 CAM 9/80 plus SVD virus UKG 27/72 which are routinely employed in the indirect sandwich ELISA for FMD/SVD virus antigen detection within the FAO World Reference Laboratory for Foot and Mouth Disease (WRL for FMD) were used. These antisera had been raised in rabbits and guinea pigs according to the methods of Have and Schjerning-Thiesen, 1984 and Ferris and Donaldson, 1984, respectively.

Monoclonal antibodies. Mouse monoclonal antibodies (mabs) to strains of each serotype of FMD and SVD virus were selected for test. The mabs to FMD virus serotypes C (strain C1, Oberbayern, mabs E2B4 and D7G2), SAT 1 (BOT 1/68, Clone 5), SAT 3 (ZIM 4/81, C14) and Asia 1 (PAK 1/54, C1) plus SVD virus (UKG 27/72, C70) were produced in-house. Other mabs against the remaining FMD virus serotypes were gifts from other FMD laboratories: O (O; Lausanne, C9) from Dr E Brocchi, Istituto Zooprofilattico Sperimentale Della Lombardia E Dell’Emilia, Via A. Bianchi, 9 - 25124 Brescia, Italy; A (A<sub>22</sub> IRQ 24/64, 18H11) from Bayer AG, BG Tiergesundheit, Biologische Produktion, Osteratherstr 1A, D-50739 Koln, Germany and SAT 2 (ZIM 07/83, 810) from Dr D Fargeaud, Botswana Veterinary Institute, Broadhurst Industrial Estate, Private Bag 0031, Gaborone, Botswana. All these mabs have been shown to be type-specific (N.P. Ferris, unpublished results). Two other FMD virus mabs (types C, 4A3 and Asia 1, 5F10) were also obtained from Dr E Brocchi, Italy, both of which react with FMD viruses belonging to all of the seven serotypes (E. Brocchi and N.P. Ferris, unpublished results).

Virus sample preparation. Inactivated, purified antigens to each of the seven serotypes of FMD virus plus SVD virus (Ferris et al., 1984) were used. Epithelial suspensions (ES) of reference samples of all seven serotypes of FMD virus plus SVD virus (as indicated in the figures) had previously been prepared in phosphate buffer (Ferris and Dawson, 1988) and had been stored at -80°C. Supernatant fluids derived from cell cultures (primary calf thyroid, IB-RS-2 or baby hamster kidney cells) inoculated with ES or cell culture grown antigens of all seven serotypes of FMD virus plus SVD virus (strains as indicated in Table 1) were selected from a collection stored at -80°C.

Blocking Buffers. Three different blocking buffers were employed depending on the particular ELISA (capture/detecting) reagent combination: i) blocking buffer 1 - PBS (pH 7.6) with 0.05% Tween 20 containing 5% skimmed milk powder ("Marvel"); ii) blocking buffer 2 - 0.85% saline with 0.02 M Tris, 0.002 M CaCl<sub>2</sub> and 0.001 M MgCl<sub>2</sub>, and 2% bovine serum albumen (pH 7.6) and iii) blocking buffer 3 - PBS (pH 7.4) with 0.05% Tween 20 and containing 10% normal bovine serum and 5% normal rabbit serum.

Indirect sandwich ELISA. The basic format for the indirect sandwich ELISA, unless otherwise stated, was as follows: 50 µl reagent volumes were used throughout; ELISA plates (Nunc Maxisorp immunoplates) were incubated for 1 h at 37°C on a rotary shaker and plates washed with phosphate buffered saline [PBS, pH 7.4] after each incubation step, except the final stage in which sulphuric acid was added to stop the substrate/chromogen reaction.

ELISA using polyclonal antisera as capture and detecting antibodies. Plates were coated with an optimal dilution of rabbit antiserum to FMD virus in 0.05 M carbonate/bicarbonate buffer, pH 9.6 and incubated in a fridge overnight at +4°C. Next, either purified virus antigen, suspensions of vesicular epithelia or cell culture supernatants were added to each well. Where applicable, antigen was diluted in PBS. After plate incubation, homologous guinea pig antiserum, diluted to the optimal concentration in blocking buffer 1 was added to each well. After plate incubation, an optimal dilution of rabbit anti-guinea pig immunoglobulins conjugated to horse radish peroxidase in blocking buffer 1 was added to each well. The plates were washed after incubation and plates blotted dry before substrate (0.05% H<sub>2</sub>O<sub>2</sub>)/chromogen (orthophenylenediamine) in citrate/phosphate buffer, pH 5.0 was added. After 15 min incubation at room temperature the reaction was stopped by adding 1.25 M sulphuric acid. The OD of each well was read by using a spectrophotometer with a 492 nm filter.

ELISA using recombinant αvβ6 as a capture ligand and polyclonal antibodies as detectors. Plates were coated with an optimal dilution of recombinant αvβ6 in a solution of 0.85% saline, 0.02 M Tris, 0.002 M CaCl<sub>2</sub> and 0.001 M MgCl<sub>2</sub>, pH 7.6 and incubated in a fridge overnight at +4°C. The following day, the coating buffer was tipped off the plate without washing with PBS and 100 µl of blocking buffer 2 was added to each well. After plate incubation for 1 h at 37°C, the blocking buffer was simply tipped off the plate, test sample added and the ELISA completed as described in the previous section. Where applicable, antigen was diluted in blocking buffer 2. After plate incubation, an optimum dilution of mab in blocking buffer 3 was added and the ELISA completed as described, except that
rabit anti-mouse, instead of rabbit anti-guinea pig, immunoglobulins conjugated to horse radish peroxidase, and diluted in blocking buffer 3, was used.

Optimisation of recombinant \( \alpha v \beta 6 \) dilution for use in the ELISA. The optimal dilution of recombinant \( \alpha v \beta 6 \) for coating immunoplates was assessed as follows. Two-fold dilution series of the integrin in saline coating buffer were made (from columns 1 to 12 of immunoplates) from undiluted CHO cell culture supernatant fluid. A fixed dilution (1 µg/ml) of purified antigen of each FMD virus serotype plus SVD virus was then added, one virus serotype for each of the 8 rows, and the test run to completion. The optimal dilution of the recombinant preparation was then chosen from the titration plot of the reactivity of recombinant dilution against the virus antigens.

Evaluation of recombinant \( \alpha v \beta 6 \) as a ligand for FMD virus. The ability of the recombinant \( \alpha v \beta 6 \) to bind FMD virus was assessed by comparing a series of immunoplates which had been coated with an optimal concentration of recombinant integrin with others which had been left uncoated. The plates were blocked after incubation with blocking buffer 2 prior to the addition of cell culture supernatants to 12 isolates of each FMD virus serotype plus SVD virus (Table 1). The two pan-reactive mabs (4A3 and 5F10) were used as detecting antibodies and the format of the ELISA was carried out as previously described for recombinant \( \alpha v \beta 6 \) as a capture ligand and mabs as detectors. The results were also compared to the reactivity of the antigens in the described ELISA procedures using rabbit antiserum as a trapping reagent in combination with either guinea pig polyclonal or mouse monoclonal antibodies.

FMD virus diagnosis by ELISA. The three strategies for the ELISA (i.e. rabbit polyclonal antibody as trapper and guinea pig polyclonal antibody as detector, recombinant \( \alpha v \beta 6 \) as trapper/guinea pig as detector and recombinant \( \alpha v \beta 6 \) as trapper/type-specific mab as detector) were compared for their ability to serotypically discriminate samples of cell culture grown antigens and epithelial suspensions of each serotype of FMD and SVD virus (plus another suspension negative for FMD or SVD virus).

Results:
Optimal recombinant \( \alpha v \beta 6 \) dilution for use in the ELISA. It was found that the unpurified CHO cell culture supernatant fluid containing the \( \alpha v \beta 6 \) protein bound the inactivated, purified viruses of all seven FMD virus serotypes but did not react with SVD virus. The results of titrations of this recombinant preparation against purified virus preparations are shown in Fig. 1A. Too high an integrin concentration proved to be inhibitory for binding virus and reactivity rapidly tailed off past an integrin dilution of 1:512 but the mid-range of the dilution series (from dilutions 1:16 to 1:256) proved effective for trapping FMD virus. Consequently, a dilution of 1:100 of the CHO cell culture supernatant fluid was chosen to coat immunoplates for subsequent examination of test antigen preparations in the ELISA.

The conclusion that FMD virus was indeed bound by the recombinant integrin was supported by the demonstration that virus preparations were prevented from binding directly to the surface of immunoplate wells treated with blocking buffer 2 prior to the antigen step (Fig. 1B). However, this treatment did not interfere with the positive reaction between integrin and FMD virus on integrin coated plates.

Evaluation of recombinant \( \alpha v \beta 6 \) as a ligand for FMD virus. The reaction of 12, cell culture grown antigens of each FMD virus serotype and SVD virus (Table 1) was evaluated in each ELISA format and the results are shown in Fig. 2 (each mini graph is entitled with the serotype of each group of 12 viruses used for the ELISA reaction). The results show that the integrin bound all FMD viruses irrespective of serotype (the plot profiles being similar between Fig. 2 sections A, B, C, E and F) but not SVD virus. The specificity of the reactions being confirmed by the inability of the viruses to bind directly to the surface of immunoplates uncoated with either rabbit antiserum or integrin but which had been first blocked with blocking buffer 2 (Fig. 2D).

Serotypic characterisation of test samples. The results of comparisons between the three ELISA formats to classify the serotype of the test samples are shown in Fig. 3 (using epithelial suspensions). Each mini graph is entitled with the virus serotype and sample strain used for the ELISA reaction. The pattern of results which were achieved using cell culture grown antigens was essentially the same.

Although the signals for the homotypic reactions were strongest, there was evidence of a degree of heterotypic cross-reactivity with some of the test samples using rabbit/guinea pig polyclonal antisera (Fig. 3A). This heterotypic cross-reactivity was further and considerably exacerbated using the integrin/guinea pig polyclonal antibody combination (Fig. 3B). Conversely, totally type-specific reactions resulted from the ELISA employing recombinant integrin as capture and mabs as detecting
reagents (Fig. 3C). No reactions were evident when utilizing integrin as the trapping reagent with the addition of SVD virus or the negative epithelial suspension.

**Discussion:**

Molecular techniques, such as RT-PCR procedures, are certain to play an essential role in the future diagnosis FMD and other vesicular diseases. The real-time, fluorogenic RT-PCR is being shown to be more sensitive than the ELISA for FMD diagnosis (Reid *et al.*, 2003; Shaw *et al.*, 2004). It has the potential to examine certain diagnostic samples such as blood, milk and other fluids, which cannot be examined directly in the ELISA and to reduce the necessity for virus isolation and amplification in cell cultures. Although the ELISA has inferior sensitivity to real-time RT-PCR, the predictive value of the test can be high if suitable samples can be collected. For example, around 90% of positive epithelium samples received during the course of the 2001 FMD outbreak in the UK were so defined at the initial stage of testing epithelial suspensions by ELISA (N.P. Ferris, unpublished results). The ELISA is also quicker, easier and cheaper to perform than the PCR. It is an assay, which is readily transferable from a reference laboratory to other FMD laboratories, the majority of which already have the necessary equipment and reagents for its use and the expertise to perform it.

The main disadvantage of the ELISA as used by the WRL for FMD and the majority of other FMD laboratories (besides not having 100% sensitivity) is that it uses FMD virus type-specific polyclonal antisera: both to trap FMD virus onto the plate and for its detection. The disadvantages of polyclonal antisera include a continual need to ensure that reagents for diagnostic use have suitable affinity for new emerging field virus strains and that each stock is of finite supply, while the replacement stock often exhibits slightly different reaction characteristics. To counter the finite supply of polyclonal antiserum reagents, type-specific mabs can be used (Brocchi *et al.*, 1986) but the problem of ensuring that the selection of panels of either individual mabs, or cocktails of several, is suitable for the recognition and serotypic discrimination of new antigenic virus strains remains.

The objective of the work reported on here was to examine the utility of recombinant integrin αvβ6 (arising from CHO cells, which have been transfected to secrete soluble integrin into the maintenance medium) for binding strains of all serotypes of FMD virus in the ELISA. This was demonstrated by testing purified FMD and SVD virus antigens in an ELISA using serum-free maintenance medium from the transfected CHO cells (as collected directly from the culture flasks, save for a clarification of the medium to remove cell debris by centrifugation) containing recombinant integrin protein as the capture reagent. This was followed by the demonstration that panels of cell culture grown antigens of each FMD virus serotype were bound by the recombinant protein while those of SVD virus were not. Although there were examples of variable optical density values for certain viruses between the different ELISA formats, most of the differences in signals were probably a reflection of the reactivity of the reagent used to detect the bound virus. For example, although the pan-reactive FMD mabs 4A3 and 5F10 react with FMD viruses of each serotype, mab 4A3 had a higher affinity for SAT 1 FMD viruses than mab 5F10 while the reverse was evident for some FMD viruses of the Asia 1 serotype.

A limitation of the conventional ELISA (using rabbit antiserum as capture antibodies and polyclonal antibodies as detectors) is that of heterotypic cross-reactions which sometimes reduce the certainty of serotype definition. Such heterotypic reactions were evident from the described experiments on serotyping both cell culture grown viruses (results not shown) and epithelial suspensions of field strains of FMD viruses (as illustrated in Fig. 3). Homotypic signals were the highest for each of the viruses examined by the ELISA using rabbit/guinea pig antibody combination with a reasonably clear distinction from the heterotypic reaction (Figs 3A). This proves to be generally the case during the course of examining submitted diagnostic samples received under the auspices of the WRL for FMD. However, problems of test interpretation occur in two situations. Firstly, high concentrations of antigens in sample preparations that have a poor antigenic match with the typing reagent may give rise to a lower optical density value in the ELISA against the homotypic reagent than would normally be expected. Consequently, the differences between homotypic and heterotypic reaction signals are reduced, lowering the confidence with which the serotype of the FMD virus is defined. Secondly, it is normal to assume that heterotypic reactions are indeed non-specific cross-reactions. However, this can be a dangerous assumption. Multiple-infected samples are uncommon but they do occasionally occur and their classification by ELISA, with or without virus isolation in cell culture, is problematic (Ferris, Oxtoby and Hughes, 1995). The combination of integrin as capture and guinea pig polyclonal antibody as detector yielded even greater heterotypic cross-reactions (Figs 3B). This was disappointing and somewhat surprising but illustrates that the integrin is not solely specific for FMD virus and that the guinea pig anti-146S sera are not as FMD virus type-specific as previously thought or hoped for. However, replacing the polyclonal reagents with mabs eliminated heterotypic reactions and yielded totally FMD virus type-specific results of the correct definition. This is not to suggest that the particular mabs used in this study are necessarily the optimum ones for routine diagnostic use (some have too narrow a range of within serotype strain recognition for use on their own). Rather the results show that the use of the recombinant protein as a capture reagent in the ELISA in
combination with appropriate mab(s) as the detector has the potential to improve upon conventional diagnostic assays using rabbit and guinea pig polyclonal antisera.

There is further potential for use of the recombinant integrin $\alpha\nu\beta_6$ protein in other FMD virus test procedures where the use of a single reagent to recognise FMD viruses of all serotypes could be advantageously exploited, e.g. in immunocapture RT-PCR, pen-side chromatographic strip-test devices and other biosensors. Additionally, the recombinant could be usefully employed in any immunoassays employed for FMD diagnosis. Those designed for characterizing the antigenicity of field virus strains (Kitching, Rendle and Ferris, 1988) and for detecting FMD virus antibody (Mackay et al., 2001) come to mind. These assays commonly use type-specific rabbit polyclonal antibody to trap virus onto the immunoplate, the efficiency of which is limited by the antisera selection.

**Conclusions:**
- The recombinant $\alpha\nu\beta_6$ protein bound all antigens of FMD virus irrespective of serotype but did not react with SVD virus
- Totally type-specific reactions resulted from the ELISA using integrin as capture and mabs as detectors
- This format has the potential to improve upon conventional FMD diagnostic assays using polyclonal antisera and could be usefully used in other immunoassays for FMD diagnosis

**Recommendations:**
- No specific recommendations

**Acknowledgements:**

This work was supported financially by the Department for the Environment, Food and Rural Affairs (project number SE1120). We thank D. Sheppard (UCSF) for generous access to $\alpha\nu\beta_6$ CHO cells and L. Weixan for biotechnological support.

**References:**


**TABLE 1. FMD and SVD virus strains used to evaluate the capacity of recombinant αvβ6 to act as a ligand**

<table>
<thead>
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<th>Virus sample no.</th>
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<th>SVD virus</th>
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<tr>
<td>O</td>
<td>A</td>
<td>C</td>
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Fig. 1.

A. Titration of CHO cell culture supernatant fluid containing soluble recombinant integrin against purified inactivated antigens of each of the seven serotypes of FMD virus plus SVD virus (at 1 ug/ml)

B. Reaction of purified inactivated antigens of each of the seven serotypes of FMD virus plus SVD virus (at 1 ug/ml) added directly onto plates previously blocked with blocking buffer 2
Fig. 2. Homotypic reactions of cell culture grown antigens of each of the seven serotypes of FMD virus plus SVD virus in ELISAs using combinations of reagents for capture/detection of virus as indicated by the captions for sections A to F.

A  Homotypic rabbit/guinea pig polyclonal antibodies

Optical density

B  Homotypic rabbit polyclonal antibodies/pan-reactive RMD virus mab 4A3

Optical density

C  Integrin/pan-reactive FMDV mab 4A3

Optical density

D  Non-coated/pan-reactive RMD virus mab 4A3

Optical density

E  Homotypic rabbit polyclonal antibodies/pan-reactive RMD virus mab 5F10

Optical density

F  Integrin/pan-reactive FMDV mab 5F10

Optical density

Virus number (1-12 for each graph)
Fig. 3. Homo- and heterotypic reactivity of epithelial suspensions of each of the seven serotypes of FMD virus (serotype and strain as indicated by the graph headings) and a negative sample (NVD, no virus detected) using combinations of reagents for capture/detection of virus (as indicated by the captions for sections A to C). Homotypic reactions as bold plots and heterotypic reactions as empty plots.

A  Homotypic rabbit/guinea pig polyclonal antibodies

B  Integrin/homotypic guinea pig polyclonal antibodies

C  Integrin/homotypic monoclonal antibodies

Virus serotype (order on each x axis: FMD virus types O, A, C, SAT1, SAT2, SAT3 and Asia 1 plus SVD strains)