



NEW DEVELOPMENTS IN FOOT-AND-MOUTH DISEASE DIAGNOSIS

R.P. KITCHING, D.K.J. MACKAY
Institute for Animal Health,
Compton, Berkshire, United Kingdom

Abstract

NEW DEVELOPMENTS IN FOOT-AND-MOUTH DISEASE DIAGNOSIS.

A variety of newer diagnostic procedures based around the use of molecular technologies are now being undertaken to further characterise the foot-and-mouth disease (FMD) virus enabling a deeper understanding to be gained of the pathogenesis and epidemiology of this disease. Such approaches have categorically identified the carrier state and highlighted the importance of carrier animals in control programmes. Use of the polymerase chain reaction provides even further insight into the carrier animal but interpretation of data has to be undertaken with caution. The role of non-structural proteins can provide further insight into an animals response to both vaccination and natural infection and could provide a basis for separation of the carrier state. Finally the pivotal role of monoclonal antibodies in all aspects of FMD research is now clear and these highly specific reagents are now being used for a variety of research and diagnostic purposes within the FMD field.

1. INTRODUCTION

The routine use of molecular biological techniques in the diagnostic laboratory has not only greatly increased the speed, specificity and sensitivity of foot-and-mouth disease (FMD) diagnostic tests, but has also greatly assisted in our understanding of the epidemiology and pathogenesis of the disease. Novel, or improved, techniques have been applied both to genomic studies, using nucleotide sequencing and polymerase chain reaction (PCR), and to antigenic studies, using expressed proteins and monoclonal antibodies, to define more precisely the antigenic nature of the virus and the immune response it provokes.

2. FMD CARRIERS

Using these novel techniques [1], it has been possible to show identity between strains of FMD virus found in African buffalo and cattle, and between strains causing outbreaks in cattle over extended periods [2,3]. For the first time there is laboratory confirmation that cattle and buffalo carrying FMD virus can initiate new outbreaks of disease. The carrier animal is defined as one from which live FMD virus can be isolated 28 days after contact with infection. This may be a fully susceptible animal which develops clinical disease and in which virus persists following recovery, or a vaccinated animal that has contact with live virus and fails to develop clinical disease, but becomes a carrier. In the carrier state, FMD virus persists in the pharyngeal region, in the presence of specific anti-FMD virus antibodies in the circulation and pharyngeal secretions. Sheep and goats are reported to carry FMD virus for up to nine months, cattle and African buffalo for up to, and possibly longer than, three and five years respectively; pigs do not become carriers, and other susceptible wildlife ungulates probably carry virus for only a relatively short period [4]. In spite of considerable research effort, the specific cells in the pharynx in which FMD virus persists, have not been identified.

The importance of the carrier animal in the epidemiology of FMD has long been assumed, and for the purposes of international trade, any animal with FMD virus antibody is considered a potential carrier. As the economic consequences of an outbreak of FMD in the European Union (EU) would be considerable, even a very small risk is unacceptable. However, until recently there was only circumstantial evidence to link outbreaks of FMD with carrier animals.

Although the EU ceased prophylactic vaccination against FMD after 1991, the option to vaccinate is retained, should slaughter and other zoosanitary measures not be effective in controlling an outbreak. A vaccine bank of concentrated FMD antigens is currently being set up on four sites within the EU. Should vaccination be used to help control an outbreak of FMD, all vaccinated, sero-positive cattle,

and sheep, would be considered potentially to have had contact with live virus, and therefore, possibly to be carriers. If these animals were not very soon slaughtered after the outbreak had been controlled, they would require constant surveillance to ensure that they did not mix with susceptible animals. It is likely that if vaccination were reintroduced, large numbers of animals would be involved, making permanent supervision impossible. In addition, the EU perceives a risk from FMD carrier animals following the extension of trading agreements to countries outside of the EU and from which live animal imports occur. Already, in March 1993, there has been an example of live cattle being brought into Italy with false certification, causing an outbreak of FMD.

However, there is no reliable technique available to distinguish between the serologically positive carrier and non carrier animal, and because of the costs of an outbreak of FMD only a 100% sensitive test would be acceptable. One hundred percent specificity would not be essential, assuming the number of false positive reactions was within acceptable limits. Because of the requirement to identify the carrier animal, much of the research into new diagnostic tests has been directed towards this aim.

The traditional method used to identify the carrier is the probang cup [5]. A scraping is made of the pharynx and anterior oesophagus and the material collected is used to inoculate susceptible cells in tissue culture. The most sensitive tissue culture system is primary bovine thyroid cells. The sensitivity of the test can be further increased by homogenising the sample with Arcton or Freon before inoculation of tissue culture. However, single sampling, even under the most suitable conditions, including those of collection and transport to the laboratory, results in less than 50% of carriers being identified. Repeat sampling improves the opportunity to isolate virus, but many carriers will still be missed.

3. POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) is a technique which amplifies a specific genome segment of virus in a diagnostic sample. It relies on prior knowledge of the nucleotide sequences flanking the region to be amplified. For the identification of FMD virus genome, a c-DNA segment is first produced from a short DNA oligonucleotide primer which is complementary to the FMD virus genome in the sample. Following this, and using an additional oligonucleotide primer complementary to the distal end of the c-DNA, a further complementary sequence is produced. These two complementary strands of DNA are then amplified through a series of primer annealing, extension and thermal denaturation in the presence of a mixture of nucleotides and thermostable DNA polymerase. Each cycle doubles the quantity of amplified segment to a level that can be detected on an agarose gel. The specificity of the amplified sequence can be confirmed with a labelled DNA probe, by restriction endonuclease digestion or by nucleotide sequencing a region or all of the product. A range of specific primers is used in the World Reference Laboratory (WRL), Pirbright which can identify all known strains of serotype A, O, C, and Asia 1. They are not yet available for the SAT serotypes.

When applied to probang samples, PCR has increased the sensitivity of the detection of carriers considerably. In one trial reported by Amarel-Doel et al. [6] in which 101 probang samples were examined, 39 were negative by PCR and tissue culture, 19 were positive by both tests and 26 were positive by PCR and negative on tissue culture. However, 17 were positive on tissue culture and negative by PCR. There appears to be non-specific inhibitors of PCR, such as blood, which can cause false negatives. The use of PCR for identifying carriers is therefore limited, although it is a valuable additional test to the traditional tissue culture technique. In addition, it could be argued that the demonstration of FMD virus genome by PCR does not necessarily indicate the presence of live virus in the sample.

Nevertheless, PCR is likely to be used more routinely as more experience with the technique is acquired, and the causes of false negative results identified. It has already been used specifically to identify strains of virus present in FMD vaccines when the live vaccine strains have not been made available to the WRL. It is also now being used to obtain directly the nucleotide sequence of strains of FMD virus present in diagnostic samples. This avoids the requirement to grow isolates on tissue culture which could result in the selection of strains better able to grow in culture or even predispose to mutation of the original strain present.

4. NON-STRUCTURAL PROTEINS

An alternative approach to identifying the carrier animal which, although indirect, would have greater application, is to develop a serological test. Current serological tests are able to distinguish animals which have had previous contact with FMD virus antigen from naive animals which have not. However, they cannot distinguish animals which have been merely vaccinated from those which have been infected, nor between recovered animals which have eliminated the virus and persistently infected carrier animals.

Conventional FMD vaccines consist of inactivated whole virus preparations with a suitable adjuvant. Vaccination therefore results in the production of antibodies to the structural proteins on the surface of the virion, and should the animal subsequently be exposed to infection these antibodies will inactivate live virus and provide protection. In principle, the immune response to infection with FMD virus differs from the response to vaccination. During the process of viral replication FMD virus is initially produced as a single polypeptide chain which is subsequently broken down into a number of structural proteins, which form the virion itself, and a number of non-structural proteins (NSP's), which have biological activity either on the FMD virus polypeptide or on the host cell (Figure 1). A number of these NSP's have been shown to be immunogenic. Theoretically therefore, the detection of antibody to NSP's should indicate infection rather than vaccination. However, in practice, trace amounts of NSP's can be found in commercial vaccines which also induce the production of antibody, particularly following repeated vaccination.

The NSP which provokes the strongest immune response and which has consequently been the best studied is the viral RNA polymerase (protein 3D, also known as the Virus Infection Associated Antigen, VIAA). This protein, like most NSP's, is highly conserved between strains and even between serotypes of FMD virus, holding out the possibility of a single serological test capable of detecting infection with any of the seven serotypes of the virus. Unfortunately a reliable, sensitive and specific assay for antibody to VIAA has proved elusive and despite the fact that the first test for antibody to VIAA was reported over 25 years ago [7], no VIAA test has yet found general acceptance. Conventional assays for antibody to VIAA use a semi-purified antigen prepared from the virus grown in tissue culture. When used for immunodiffusion, the antigen results in a test with poor sensitivity and specificity [8] and, when used in ELISA, there are problems of inadequate reproducibility (WRL, unpublished findings).

Molecular biological techniques have now been applied to clone the genes coding for the NSP's into a number of different vector systems and work is underway both at the WRL and other laboratories in Europe and South America to explore the potential of these expressed products as antigens in ELISA. The potential use of these antigens is considerable as it should be possible to develop a test which can detect infection with any serotype in a single assay and differentiate animals which have been infected from those which have been vaccinated. Furthermore it should be possible to identify the carrier animal, as animals which have been infected and subsequently eliminate the virus produce a different spectrum of response to NSP's from animals which go on to become carriers. An ELISA using an expressed protein has the added advantage that the antigen presents no biological hazard.

Initial work using an expressed VIAA [9] was disappointing as the test had poor specificity and hence could only be used as a screening test to evaluate whether or not a population of animals had been exposed to FMD infection. Subsequently, expressed proteins have been used successfully in radioimmunoprecipitation [10] and immunoblotting studies [11, 12] to differentiate infected from vaccinated animals and also tentatively to differentiate carriers from non-carriers. However, neither of these techniques is suited to screening the large numbers of animals which would require testing should ring vaccination ever be used to contain future outbreaks within the EU. For this purpose it would be essential to use ELISA. At the WRL the major NSP's have been expressed in *E coli* as fusion proteins attached to glutathione s-transferase (GST). Following purification, and either with or without cleavage from the GST, these recombinant NSP's are recognised by immune sera from animals infected with a range of serotypes of FMD virus. When used in a simple indirect ELISA, recombinant 3D is able to differentiate infected from naive cattle. The sensitivity of the test is only slightly lower than the conventional liquid phase blocking ELISA of Hamblin et al. [13] and the specificity is approximately 95%. Vaccinated animals, especially those which have received more than one dose of vaccine, give a

positive reaction, as can up to 5% of normal bovines. It therefore appears that the recombinant (and possibly the native) 3D protein shares epitopes with other antigens which are encountered by cattle, perhaps the polymerase of other enteroviruses. Further work is required to characterise the recombinant NSP's and the immune response against them. A range of ELISA techniques are currently being evaluated with the intention of producing ELISA's for routine diagnostic use which are capable of detecting antibody to FMD virus NSP's.

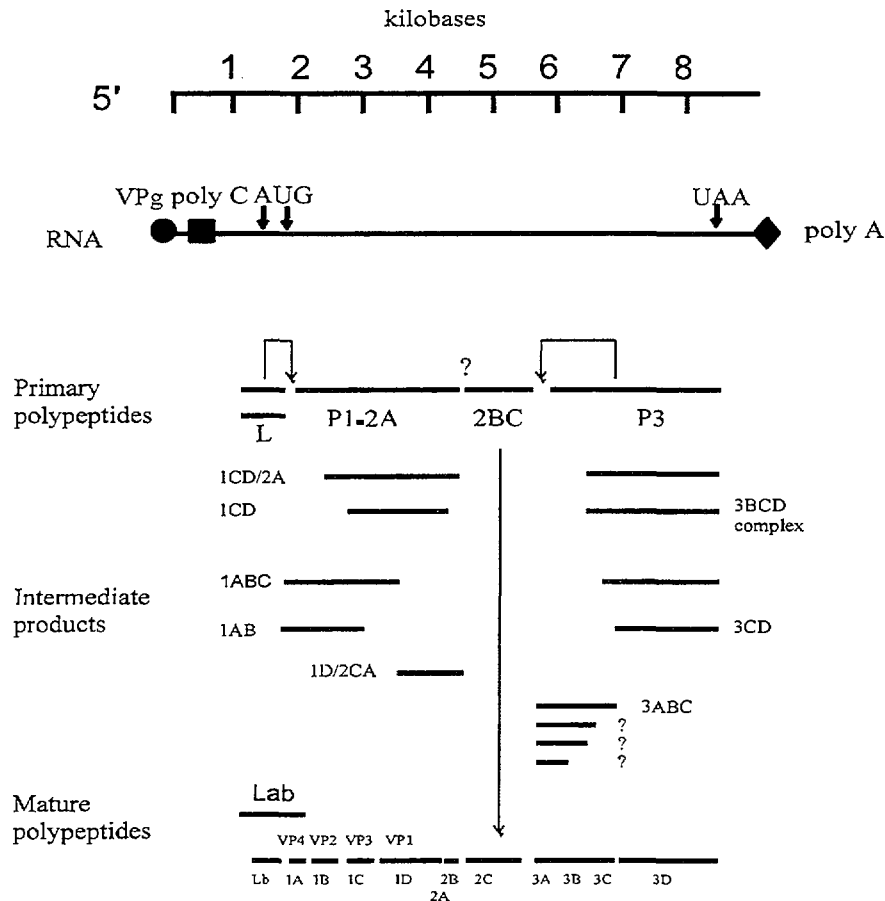


FIG. 1. Genomic organization and proteolytic processing of the FMDV polyprotein.

5. MONOCLONAL ANTIBODIES

Monoclonal antibodies (Mabs) are the products of a clone of identical, immortal antibody-producing cells. They consist of a population of identical antibodies all of which have specificity for one single, definable epitope (antigen binding site). This contrasts with polyclonal antisera, produced by immunisation of animals, which consist of mixed populations of antibodies against a range of different epitopes. Mabs against FMD virus have been produced since the 1980's and have provided useful information about the structure and function of the virus. Only now are they starting to be more widely applied to improve FMD diagnosis.

Mabs can be used in ELISA to detect either antigen or antibody. Mabs have been applied with some success to FMD antigen detection ELISA's with the aim of increasing specificity. A serotype-specific Mab-based antigen detection ELISA currently in use at the Istituto Zooprofilattico Sperimentale, Brescia, Italy [14], relies on a mixture of at least 3 different Mabs against each of the serotypes O, A and C to detect FMD virus in clinical samples. The use of multiple Mabs has the advantage that it is less likely that a field virus will fail to possess at least one of the epitopes recognised by the antibodies. If a Mab can be produced against an epitope shared by all the serotypes of FMD virus it should be possible

to detect virus of any serotype with a single assay. Smitsaart et al. [15] reported an assay using a Mab reactive against an epitope on the 12S sub-unit of FMD virus which was shared between 6 of the 7 serotypes of FMD virus. Work is continuing to produce a Mab reactive against an FMD virus-specific epitope which is sufficiently stable to form the basis of a diagnostic test.

In the FMD virus antibody detection ELISA Mabs can be used either to trap the FMD virus antigen or, more usually, to compete with test sera for binding to antigen. Due to the unique specificity of Mabs, competition assays are able to differentiate serological responses to antigens which are very closely related, such as antibodies to rinderpest and peste des petits ruminants [16]. Competition [14] and complex-trapping-blocking [17] ELISA's using combinations of serotype-specific Mabs have been developed. As described for a non-serotype specific antigen detection ELISA, the identification of an epitope shared between all of the seven serotypes of FMD virus could be the basis of a non-serotype specific competition ELISA able to detect antibody to any strain of FMD virus. Due to their highly conserved nature, epitopes on the NSP's of the virus are the most likely candidates for such a site. However, the search is complicated by the fact that antibody to NSP's is not neutralising. Therefore it is not possible to raise Mab escape mutants and sequence them to identify the sites at which the Mabs bind. Alternative approaches are required such as scanning libraries of overlapping peptides corresponding to the predicted amino acid sequence of the protein to identify the residues important in Mab binding.

Mabs now also play an important role in characterising field, vaccine and reference strains of FMD virus. Panels of Mabs have been produced and characterised against strains of FMD virus types O, A, C & SAT 2. Many Mabs have also been produced against strains of other serotypes and characterised to varying degrees. Attempts have been made over several years to establish a bank of FMD Mabs at the WRL, Pirbright with limited success and a new initiative has recently been launched under the auspices of the Community Reference Laboratory section of the WRL. These panels of Mabs are used to characterise the major epitopes of strains of FMD virus. Comparisons between field and vaccine strains, or between seed strains and final vaccines strains, give precise information about the similarity of the epitopes examined. Once sufficient information is available about which of these epitopes are important for protection and how this relates to Mab binding, it should be possible to predict on the basis of a Mab profile whether a vaccine strain is likely to give protection against a particular field strain. Furthermore our knowledge of the link between the genotype (nucleotide sequence) and phenotype (Mab profile) of viruses increases as more and more strains are examined. This holds the prospect of eventually being able to predict important nucleotide sequence changes on the basis of Mab profiles, which would save the considerable time and expense of sequencing large numbers of isolates.

ACKNOWLEDGEMENT

The authors are grateful to Dr. J. R. Crowther for the use of Figure 1.

REFERENCES

- [1] KITCHING, R.P., The application of biotechnology to the control of foot-and-mouth disease virus, *Br.Vet. J.* **148** (1992) 375-388.
- [2] DAWE, P.S., FLANAGAN, F.O., MADEKUROZWA, R.L., SORENSEN, K.J., ANDERSON, E.C., FOGGIN, C.M., FERRIS, N.P., KNOWLES, N.J., Natural transmission of foot-and-mouth disease virus from African buffalo (*Syncerus caffer*) to cattle in a wildlife area of Zimbabwe, *Vet.Rec.* **134** (1994) 230-232.
- [3] HARGREAVES, S.K., OIE Scientific Conference on the Control of Foot-and-Mouth Disease, African Horse Sickness and Contagious Bovine Pleuropneumonia, OIE, Paris, France, (1994) pp 9-10.
- [4] SALT, J.S., The carrier state in foot-and-mouth disease - an immunological review, *Br.Vet. J.* **149** (1993) 207-223.
- [5] SUTMOLLER, P., GAGGERO, A., Foot-and-mouth disease carriers, *Vet. Rec.* **77** (1965) 968-969

- [6] AMAREL-DOEL, C.M.F., OWEN, N.E., FERRIS, N.P., KITCHING, R.P., DOEL, T.R., Detection of foot-and-mouth disease viral sequences in clinical specimens and ethyleneimine-inactivated preparations by the polymerase chain reaction, *Vaccine* **11** (1993) 415-421.
- [7] COWAN, K.M., GRAVES, J.H., A third antigenic component associated with foot-and-mouth disease infection, *Virology* **30** (1966) 528-540.
- [8] McVICAR, J.W., SUTMOLLER, P., FMD: The agar gel diffusion precipitin test for antibody to virus-infection-associated (VIA) antigen as a tool for epizootologic surveys, *American Journal of Epidemiology* **92** (1970) 273-278.
- [9] VILLINGER, F., MUELLER, H.K., BRUCKNER, L., ACKERMANN, M., KIHM, U., Antibodies to FMD virus infection associated (VIA) antigen: use of a bio-engineered VIA protein as antigen in an ELISA, *Veterinary Microbiology* **20** (1989) 235-246.
- [10] TESAR, M., BERGER, H-G., MARQUARDT, O., Serological probes for some FMD virus non-structural proteins, *Virus Genes* **3** (1989) 29-44.
- [11] NEITZERT, E., BECK, E., DE MELLO, P.A., GOMES, I., BERGMANN, I.E., Expression of the aphthovirus RNA polymerase gene in *Escherichia coli* and its use together with other bioengineered non-structural antigens in detection of late persistent infections, *Virology* **184** (1991) 799-804.
- [12] BERGMANN, I.E., DE MELLO, P.A., NEITZERT, E., BECK, E., GOMES, I., Diagnosis of persistent aphthovirus infection and its differentiation from vaccination response in cattle by use of enzyme linked immunoelectrotransfer blot analysis with bio-engineered non-structural viral antigens, *American Journal of Veterinary Research* **54** (1993) 825-831.
- [13] HAMBLIN, C., BARNETT, I.T.R., HEDGER, R.S., A new enzyme linked immunosorbent assay (ELISA) for the detection of antibodies against FMD virus, 1. Development and method of ELISA, *Journal of Immunological Methods* **93** (1986) 115-121.
- [14] BROCCHI, E., DE SIMONE, F., BUGNETTI, M., GAMBA, D., CAPUCCI, L. Report of the Research Group of the Standing Technical Committee of the European Commission for the Control of FMD, Lindholm, 1990 pp. 83-88 (Appendix 14).
- [15] SMITSAART, E.N., SAIZ, J.C., YEDLOUTSCHNIG, R.J., MORGAN, D.O., Detection of foot-and-mouth disease virus by competitive ELISA using a monoclonal antibody specific for the 12S protein subunit from six of the seven serotypes, *Veterinary Immunology and Immunopathology* **26** (1990) 251-265.
- [16] ANDERSON, J., MACKAY, J.A., The detection of antibodies against peste des petits ruminants virus in cattle, sheep and goats and the possible implications to rinderpest control programmes, *Epidemiology and Infection* **112** (1994) 225-231.
- [17] VAN MAANEN, C., A complex-trapping-blocking ELISA using monoclonal antibodies and detecting specifically antibodies directed against foot-and-mouth disease types A, O and C, I. Method and characteristics, *Veterinary Microbiology* **24** (1990) 171-178.