Detection of IgG against the Foot-and-Mouth Disease Virus (FMDV) non-structural polyprotein 3ABC in cattle and sheep


BBSRC Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Woking, Surrey GU24 0NF

Summary

Results from in-house assays to detect cattle or sheep IgG against 3ABC were compared with those from a commercially available test kit in naïve or infected animals, and those first vaccinated then challenged with foot-and-mouth disease virus (FMDV) type O. Bovine IgG\textsubscript{1} could be detected 7 days post infection or challenge (dpi or dpc) using the in-house method (3 days after neutralising antibodies) but only after 10 days using the kit. Ovine IgG\textsubscript{1+2} was detected by the in-house method as early as 10-12 dpc (an average 4 days after neutralising responses) and 2.5 days before the commercial method.

When two steers were exposed to aerosols from donor pigs infected with O UKG 2001, one appeared uninfected while the other reacted in the in-house assay against 3ABC after 12 days. In a similar experiment using donor sheep, it was not until 28 dpc that both steers became positive. With the in-house test, sheep left in contact for 11 days with donors of the same species reacted against 3ABC 12 dpc but those exposed to aerosol only after 20 days. Eight vaccinated then challenged cattle were positive using the in-house test at 10 dpc as opposed to only three, which all showed clinical signs, with the test kit. In sheep given a low potency vaccine that protected against disease there was good agreement between post-challenge virus replication and reactions with the in-house 3ABC assay by which four animals were positive but only one reacted in the commercial assay. In the unvaccinated but challenged control group, the kit showed responses three days later than the in-house test. Amongst 380 ovine field samples, sensitivity was 77% among VNT positive sera with the in-house assay but only 13% using the commercial product.

Introduction

If FMD is suspected to have been transmitted to species in which infection is frequently subclinical such as sheep, widespread serological surveillance may be required in order to identify animals which have been exposed to the virus and developed antibodies. Several classes of immuno-globulins such as IgG may be produced, including those that neutralise, depending on the time which has elapsed since infection (Mulcahy et al., 1990; McCullough et al., 1992; Salt et al., 1996).

The virus neutralisation test (VNT; OIE Manual of Standards, 2000) is considered definitive in determining the antibody status of livestock, whether they are suspected of having been infected or for certification prior to international trade. However, it can only detect immuno-globulins against the capsid or structural proteins of the virus. If animals subjected to serosurvey are unvaccinated, previous infection is readily detected by tests for structural antibodies. However, if vaccination is introduced at any stage to control the spread of FMD,
positive reactors may have developed antibodies in response to either infection and/or immunisation. In the case of animals traded across international borders, there may be uncertainty over both their vaccination and infection status. Therefore, vaccination may have considerable implications for the strategy involved in serological diagnosis. Taken together with the fact that the process of identification of individual stock can be difficult in some species (e.g. sheep) which may not remain tagged for long periods, it becomes imperative to distinguish between such antibodies. It is also important to recognise that vaccinated animals may also become infected, and cattle and sheep which recover from infection, whether or not they have also been immunised, may become carriers (Burrows, 1966; Sutmoller and Gaggero, 1965) and thus be a potential source of new infection.

In addition to capsid proteins, certain non-structural (NS) ones are also produced during the process of infection by FMD virus and against which immuno-globulins may be formed. Though produced by viral replication, and hence initially present in all virus harvests, NS proteins are later removed during the purification process used in the formulation of modern vaccines and it is unlikely these would elicit such antibodies. Several ELISAs have been developed to differentiate infection from vaccination by the detection of antibodies against a number of distinct NS proteins (De Diego et al., 1997; Mackay et al., 1998; Sorenson et al., 1998) expressed in either *E. coli* or Baculovirus. Perhaps the most reliable single NS indicator is the poly-protein 3ABC which appears to produce conclusive evidence of previous infection (Mackay et al., 1998), whether or not the animals have also been vaccinated. Antibodies against 3ABC have been detected up to 395 days post infection in both cattle and sheep (Sorenson et al., 1998). This paper compares the results obtained with sera collected from naïve, infected (including field sera) and vaccinated then infected cattle and sheep in two experimental assays and a commercial kit that detects the IgG class of antibody against 3ABC.

**Materials and Methods**

Sera submitted for import/export purposes from healthy cattle and sheep in the UK and other European countries prior to the UK outbreak of FMD in 2001 were used to represent naïve populations in the in-house assays. Some of these sera had given inconclusive antibody reactions but were shown to be negative on re-sampling.

Samples were also obtained from cattle and sheep used in the transmission experiments described later (Aggarwal et al., 2002, Cox et al., in preparation) and were used to compare performance of the assays below. In addition, 380 field sera (from a total submission of 815) collected from UK sheep as part of the sero-surveillance activities during the 2001 disease outbreak of FMDV type O were used to assess sensitivity.

Poly-protein 3ABC expressed in *E. coli* (pMF21; Flint, 1995) was produced by the method of De Diego, et al., (1997) then 200µg used to immunise rabbits at four sites sub-cutaneously. Animals were boosted with the same dose after 28 days (P.Davies, personal communication). Sera were tested by checkerboard titration in the in-house indirect sandwich ELISA for activity (see protocol below; all volumes 50µls):

**Indirect Sandwich ELISA**

1) F96 Maxisorp Nunc Immuno plates were coated with an optimal dilution of rabbit anti-3ABC serum overnight at room temperature (RT) and stored at +4°C for up to two weeks.
2) Before use, plates were washed three times with phosphate buffered saline (PBS; pH 7.2-7.6). Each was divided into sections of three columns, the first of which received buffer only (PBS + 0.05% Tween 20 (PBST) + 3% commercial unsweetened, organic soya milk + 1% normal horse serum) while an optimal dilution of 3ABC in buffer was added to the next two columns. Plates were incubated at 37°C for 1 hour. Meanwhile, buffer + 1% E.Coli sonicate was used to make a 1/200 dilution (Mackay et al., 1998) of each test serum. Under these conditions an OD of 2.2-2.4 should be obtained from highest reacting 21 day post infection sera (dpi), one of which (or dilution of) was selected that gave approximately 1.5 OD at 492nm. This was further diluted in normal serum of the same species (used as negative control) to provide a weak, borderline (M.Forsyth, personal communication) positive which should produce a consistent weak/strong positive ratio (w/s).

3) Plates were re-washed then the test and control serum dilutions added to three wells of a row (i.e. to two wells containing 3ABC and one without) and incubated as above.

4) Plates were washed again then an optimal dilution of mouse anti-bovine IgG\textsubscript{1} (Serotec, UK; MCA 627) or anti-goat/sheep IgG\textsubscript{1+2} (Sigma, UK; G-2904) added in buffer and incubated at 37°C for 1 hour. 

5) After re-washing, an optimal dilution of anti-mouse immunoglobulins/HRP conjugate (Dako, Denmark; P0260) was added and incubated as above.

6) The plates were washed again to include a thorough handwash which flooded the plate to remove excess conjugate. The reaction was then developed by addition of the chromogen, 5.05mM ortho-phenylene-diamine dihydrochloride (Sigma, UK; P8412) and substrate, 4.4mM (0.015%) H\textsubscript{2}O\textsubscript{2} in phosphate-citrate buffer (Sigma, UK; P4809). Colour was allowed to develop at RT for 15 minutes and the reaction stopped by addition of 1.25M H\textsubscript{2}SO\textsubscript{4}. Optical density was measured at 492nm.

7) Results for test and control sera were processed as follows: The mean of the two wells with 3ABC minus that containing only buffer was calculated. Then for each plate, the ratio of the test sample OD and weak positive control was divided by that obtained for the strong positive, resulting in the test/positive or t/p ratio. This corrected for plate to plate and between test variation. Test sera giving t/p ratios higher than or equal to that of the weak positive control on the same plate were considered positive. Occasionally, control sera were added twice and the median calculated.

**Commercial Test Kit**

The FMD-3ABC bo-ov Chekit (Bommeli Diagnostics, Switzerland) was used to detect IgG in cattle and sheep. Colour was allowed to develop at RT until approximately 0.8 OD at 405nm was obtained for the 1/100 dilution of the positive control (which was titrated to include 1/200 and 1/400 in every test) and 0.1 for the negative. Results were expressed as a percentage of the kit positive control using a macro program (D.Rebeski, Bommeli, personal communication). The appropriate species of control sera used for the experimental assays were always included. Sera were considered ambiguous if the 1/100 dilution gave 20-30% “percent positive” (pp) and unequivocally positive if >30%.

**Virus Neutralisation Tests (VNT), Solid Phase Competition (SPC) ELISA, Probang Sampling/Virus Isolation and Real Time Polymerase Chain Reaction (RT-PCR)**

Neutralisation tests were carried out as prescribed in the OIE Manual of Standards (2000) while the competition ELISA was performed according to Paiba et al., (in preparation). Probangs were collected and tested for the presence of live FMDV by the method of Ferris and Dawson (1988). RT-PCR was carried out as described by Reid et al., (2001).
Results

Negative sera

T/P ratios found in the in-house assays amongst naïve UK and other European cattle and sheep are presented in Fig 1. A “doubtful” range was defined with lower cut-offs of 0.04 and 0.09 respectively (corresponding to 98% specificity in both species). Upper cut-offs were determined by the w+/s+ ratio, which in bovines ranged from 0.14-0.21 while in sheep, 0.18-0.26 was acceptable.

Cattle inoculated with O1 BFS 1860 (UK, 1967)

Three steers inoculated intra-dermolingually all showed clinical signs at 2 dpi and demonstrated live virus in probang samples at 3-5 dpi. UH 28 and 29 became carriers while UH 27 did not. All were VNT positive at 4 dpi. Two animals reacted positively in the in-house assay against 3ABC at 7 dpi, the other at 10 dpi (Fig 2) while the commercial kit produced affirmative results in all three cattle only at 10 dpi.

Sheep exposed to aerosols from pigs infected with O UKG 2001

Of three animals, UI 69 and 70 showed clinical signs at 8 dpc, and UI 71 at 6 dpc (Aggarwal et al., 2002). UI 69 became positive by VNT at 10 dpc, UI 70 at 6 dpc, and UI 71 after 8 days. IgG against 3ABC was detected in UI 69 after 19 days in both 3ABC tests. However, UI 70 was positive at 12 dpc using the in-house method but only after 19 days with the kit, and UI 71 reacted after 10 and 12 days respectively (Fig 3). Real time PCR revealed viral RNA in a probang from UI 69 at 8 weeks post challenge (results not shown).

Cattle exposed to aerosols from pigs and sheep infected with O UKG 2001

Of two animals exposed to pig aerosols, one appeared not have been infected while the other, UI 62 was clinically positive and reacted in the VNT at 6 dpc. UI 64 and 65, exposed to sheep aerosols showed signs of FMD only after 17 and 14 days (Aggarwal et al., 2002) and were both VNT positive at 20 dpc. UI 62 was positive in the in-house assay against 3ABC after 12 days but not until 19 days using the commercial test. Cattle exposed to infected sheep all reacted at 28 dpc in both tests except UI 64 which gave only ambiguous results in the kit (Fig 4).

Sheep exposed to aerosols from, or in contact with, sheep infected with O UKG 2001

Three animals (UI 72-4) left in-contact with sheep previously infected, developed clinical FMD faster (after 8-12 days) than the two (UI 66 and 67) which only received aerosols (Aggarwal et al., 2002) though most became VNT positive after 16 days. The in house test showed 3ABC antibodies as early as 12 dpc amongst animals in-contact but after 20 days if only exposed to aerosol. The kit revealed IgG as early as 16 dpc in-contact but only the beginning of a response in the aerosol group at 28 dpc (Fig 5). UI 72 and 73 were viral RNA positive after 8 weeks (results not shown).
O1 Cattle Vaccine Potency Test

Three groups of five steers were given different dilutions of a commercial vaccine, and there were two unvaccinated controls. Eleven of those immunised had developed a neutralising antibody response at 21 days post vaccination (dpv), though only seven showed protective levels i.e. approximately log_{10} 1.4-5 (P.Barnett, personal communication). Animals were challenged at this point with 10^5 TCID-50 administered by tongue inoculation. Only four cattle were kept beyond 10 dpc – amongst these, live virus was isolated from probangs at 28 dpc in all, clinical signs being observed in UM 64 and 72 but not in UM 60 or 69. Overall, eight were positive in the in-house 3ABC assay at 10 dpc but only three using the commercial assay (all having shown disease), though another two were very nearly so (Table 1). Two reactors in the in-house assay (UM 63 and 68) gave t/p ratios of 0.45 and 0.83 but were negative using the commercial test.

O1 Sheep Vaccine Potency Test:

A group of seven animals were immunised with an experimental low potency vaccine which gave protection from clinical disease and reduced but did not eliminate virus replication. Together with seven unvaccinated controls, all were challenged after 14 days by aerosol exposure from infected pigs. VNT responses had developed in only three of those vaccinated by 10 dpv and were slightly changed at 4 dpv. All controls except one showed virus replication at 2 dpc (results not shown), all were positive using the in-house 3ABC test by 11 dpc but with the commercial assay, they generally reacted only at 14 dpc. In the vaccinated group, five sheep were probang positive, and four reacted against 3ABC in the in-house test but only one using the kit. Sensitivity of the latter could be enhanced by using the in-house ovine weak positive as the diagnostic indicator, which generally fell in the 10-15% percent positive range.

Sheep field sera from UK 2001 FMD outbreak

Of 380 sheep sera collected as part of the sero-surveillance activities during the UK FMDV type O epidemic and screened using the SPC ELISA, those giving ≥50% inhibition (70; 18%) were re-tested using the VNT when 30 were positive (8%; Table 3) according to the criterion in the OIE Manual of Standards (2000). Of the VNT reactors, 23 (77%) were positive using the in-house assay but only 4 (13%) with the commercial test.

Discussion

In addition to robustness and reliability, an important requirement of any veterinary diagnostic test kit is high specificity since litigation may be initiated if false positives are encountered and animals are slaughtered unnecessarily. The lack of a confirmatory test is also a reason for requiring a high specificity. However, specificity should not be achieved at the expense of sensitivity; rather a balance should be arrived at, best befitting the product’s intended use and, taking into account other assays or strategies which may be used. One major use of tests to detect antibodies against the non-structural proteins of FMD, including any resulting commercial product, is in conjunction with those for measuring structural responses in sero-surveillance of livestock in which overt symptoms may be mild or absent altogether, such as sheep. This may be required following vaccination during or after an outbreak in otherwise FMD-free countries. During the epidemic of FMD in the UK in 2001, millions of sheep sera were tested for structural antibodies using the solid-phase competition
ELISA (Paiba et al., in preparation), positives being confirmed by VNT. As no vaccination took place, non-structural tests were not required.

However, in countries where immunisation is practised in response to a disease outbreak, not only animals from herds proven infected during the epidemic may be slaughtered but also, at a later stage, those which have been vaccinated. Any use of such prophylaxis on a large scale could have profound implications for serology, especially as the UK outbreak saw a watershed in attitudes towards mass slaughter of livestock. It is also very important to distinguish between immunisation and infection in animals that have crossed international borders illegally or whose history of exposure to FMDV antigen is otherwise uncertain.

The in-house method used to detect cattle or sheep IgG, of which there are two main sub-classes, against the non-structural polypeptide 3ABC of FMD proved considerably more sensitive than the commercial assay. IgG\textsubscript{1} could be detected experimentally 3 days later than the virus neutralising response but 3 days earlier than the test kit in inoculated cattle. In sheep, where the moment of infection was less well defined, detection of IgG\textsubscript{1+2} with the in-house assay was, on average, 4 days later than a positive VNT result, but 2.5 days earlier than with the commercial product. The detection of waning antibodies was also poor – three sheep infected by pig aerosol of O UKG 2001 were still positive experimentally after 214, 79 and 44 days, whereas using the commercial test, affirmative diagnoses were achieved only up to 176, 44 and 25 days respectively (not shown in results).

In vaccinated then challenged animals, eight cattle were found positive by the in-house method but only three which showed clinical signs reacted in the kit. Moreover, two found unequivocally positive in the in-house assay tested negative with the kit. In sheep given a low potency experimental vaccine which gave protection against disease but did not entirely prevent virus replication, there was good agreement between isolation of live virus and the appearance of 3ABC antibodies using the in-house test, but only one sheep was positive with the commercial assay. Amongst controls, the experimental test detected IgG 11 dpc while the kit only revealed antibodies in most animals at 14 dpc.

The manufacturers claim the Chekit FMD-3ABC bo-ov assay has an underlying specificity of 99.95 and 100% for cattle and sheep respectively. However the in-house indirect sandwich ELISA, which appears to possess nearly 100% specificity with bovine sera is still considerably more sensitive than its commercial counterpart. This may partly be due to its targeting of the IgG\textsubscript{1} isotype, which is generally produced in higher quantities than IgG\textsubscript{2} in cattle infected with virulent strains or those vaccinated then challenged (Mulcahy et al., 1990). However, the in-house ovine assay for IgG\textsubscript{1+2} – the isotypes detected by the commercial product – also appeared considerably more sensitive than the kit. Sensitivity could be enhanced somewhat in the commercial test by basing diagnosis upon the performance of the ovine weak positive control which was demonstrated to be close to borderline. This generally fell in the 10-15% percent positive range, but even re-setting the cut-off to this level still resulted in poor sensitivity compared to the in-house assay. Assuming the recommended serum dilution of 1/100 is the most appropriate as regards diagnostic potential, the problem may well lie with the conjugate, the nature of which is unknown. However, if problems associated with sensitivity can be overcome, the kit’s inherent robustness and high reproducibility will make it a powerful diagnostic tool.
References


TABLE 3: EXAMINATION OF 380 SHEEP FIELD SERA FROM A TOTAL SUBMISSION OF 815 DURING UK 2001 EPIDEMIC OF FMDV TYPE O

ALL INITIALLY SCREENED BY COMPETITIVE ELISA - THOSE WITH $\geq 50\%$ INHIBITION (70) THEN TESTED BY VIRUS NEUTRALISATION TEST (VNT)

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<th>Test Type</th>
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<td>POSITIVE BY VNT ($\geq 1/45$)</td>
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</tr>
<tr>
<td>INCONCLUSIVE (1/16-1/32)</td>
<td>10</td>
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IN-HOUSE ASSAY FOR SHEEP IgG AGAINST 3ABC

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<td>23 (77%) POSITIVE + 4 (13%) DOUBTFUL</td>
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<tr>
<td>OF VNT INCONCLUSIVES</td>
<td>5 (50%) POSITIVE + 2 (20%) DOUBTFUL</td>
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BOMMELI 3ABC CHEKIT

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