Appendix 66

EVALUATION OF A LATERAL FLOW DEVICE FOR THE PEN-SIDE DIAGNOSIS OF FOOT-AND-MOUTH DISEASE

N. P Ferris[1], A. Nordengrahn[2], G. H Hutchings[1], S. M Reid[1], D. P King[1,] K. Ebert[1], D. J Paton[1], T. Kristersson[2], E. Brocchi[3], S. Grazioli[3] and M. Merza[2]*

[1] BBSRC Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Woking, Surrey GU24 0NF
[2] Svanova Biotech AB, Uppsala Science Park, Glunten, S-751 83 Uppsala, Sweden
[3] Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna Via Bianchi, 9 – Brescia, Italy

ABSTRACT

A lateral flow device (LFD) for the pen-side diagnosis of FMDV has been developed using a monoclonal antibody (Mab 1F10) and evaluated in the laboratory for its sensitivity and specificity for the detection of FMDV. Suspensions of 1288 vesicular epithelia and two vesicular fluids from worldwide suspect cases of FMD were evaluated by the LFD. The collection represented 304 samples of different geographical origin and antigenic and molecular variation within each of the FMDV serotypes and 986 samples in which FMDV had not been detected. Additionally, five samples containing viruses of the other vesicular diseases of SVD and five of VS plus seven from true-negative samples were also tested. Simple sample homogenisers (a disposable pellet pestle with microtube and a plastic rod with disposable bijou) were evaluated for their potential abilities to prepare epithelial suspensions under field conditions for LFD use.

The LFD detected antigens of FMDV of wide diversity of all seven serotypes but weaker reactions were often evident with viruses of type SAT 2. The diagnostic sensitivity and specificity of the device at 84% and 99%, respectively, compared favourably to 85% and 99.9% of the antigen ELISA. Both the simple sample homogenisers yielded suspensions which reacted in the LFD to a similar degree to those arising from routine pestle and mortar preparation.

The results illustrate that the 1F10 LFD has the potential of providing a specific, simple, cheap and disposable FMD diagnostic test that would be capable of providing results within minutes of taking a clinical sample and which could be used on a suspect premises by a veterinarian in support of his clinical analysis. A prototype FMDV type SAT 2 specific LFD is currently being evaluated to complement the 1F10 device to decrease the likelihood of SAT 2 FMDVs going unrecognised.

1. INTRODUCTION

In the event of an outbreak of FMD in a previously FMD-free country, diagnosis is dependent on the early recognition of signs of disease by the farmer and rapid reporting to the relevant veterinary authorities to enable the clinical symptoms to be evaluated. This is followed by the submission of samples to a reference laboratory for confirmatory tests for the presence of FMD virus, antigen and genome. The availability of an objective 'point-of-care' or 'pen-side' diagnostic test would have the advantage of providing support to veterinary clinical judgment in the first instance and could reduce the time taken for test confirmation in secondary cases of disease. In FMD-endemic areas, the period between reports of disease and collection and dispatch of samples to a laboratory for disease investigation can be protracted, allowing for the possibility of sample degradation, and FMDV is very often not detected in received submissions (A. Naci Bulut, personal communication). Long distances between the sample site and the laboratory can delay the diagnostic result, which can hinder the effectiveness of local actions in the face of outbreaks. On other occasions, episodes of disease might go uninvestigated, creating uncertainty as to the true FMD status of a region. In such situations, and additionally where FMDV of more than one serotype might be circulating, the availability of a test that could be performed at the site of a suspected FMD outbreak would help in the selection of appropriate material that need be sent to the laboratory for diagnostic serotyping

and further characterization such as virus sequencing and vaccine matching analyses. In other endemic regions, where the animal health status and productivity is low, FMD control is not considered a priority but the availability of a simple and inexpensive field test might increase FMD awareness and improve epidemiological information.
The laboratory validation of a LFD using an FMDV pan-reactive Mab for the pen-side diagnosis of FMD is presented.

2. MATERIALS AND METHODS

2.1 Monoclonal antibody (Mab) for use in the lateral flow device (LFD)

A Mab, designated 1F10 and produced at IZS, Brescia against the FMD type O virus strain UKG 31/2001 by methods as described previously (Brocchi et al., 1986), was chosen for incorporation into a LFD for the full laboratory validation study.

2.2 1F10 LFD assembly

The Mab 1F10 was coupled to 40 nm colloidal gold particles using a proprietary method and the conjugate was stored at 4 ^{o}C until use. The Mab 1F10, dissolved in 0.5 M Tris buffer, pH 8.0 to a final concentration of 1.8 mg/ml, was applied to the nitrocellulose membrane (Hi-flow membrane, Millipore, USA) using Bio-Dot air-brush equipment (Bio-Dot, UK). Fifty microliters of the Mab solution were added per 30 cm of membrane. Rabbit anti-mouse antibodies (DAKO, Denmark) were applied (control band) at a concentration of 1.8 mg/ml parallel to the Mab line (test band). The membranes were then dried at 37 ^{o}C for 45 min and stored in sealed foil sachets until use. The gold conjugate was applied to the filter (Whatman, UK) using Bio-Dot air-brush equipment (Bio-Dot, UK) at a volume of 1 µl per mm filter. The filters were dried at room temperature for 45 min and then stored in sealed foil sachets until required. The filter was overlaid onto the base of the nitrocellulose membrane, parallel to the control and the antibody bands, stuck to the membrane with adhesive and cut into 0.8 cm wide strips. The fibre/membrane strips were assembled into a device as described previously (Brüning et al., 1999) so that the filter constituted the sample pad situated above a nitrocellulose membrane strip.

2.3 Test samples

Twelve hundred and eighty seven vesicular epithelia and a vesicular fluid from suspect cases of FMD from around the world that had been submitted to the FAO WRL for FMD from 1965 to 2008 (plus one other epithelium and a fluid sample examined under field conditions) were evaluated by the LFD. The samples were selected from a collection representing different geographical origins and antigenic and molecular variation within each of the FMDV serotypes (n=304) as well as others in which FMDV had not been detected (n=986; classified as 'no virus detected'). In addition, samples containing viruses of the other vesicular diseases of SVD (n=5) and VS (n=5) and from samples collected from naïve animals (n=7) were also tested.

2. 4 Virus detection tests

The majority of tests for virus detection were undertaken at the time of sample receipt and encompassed the following procedures. Virus isolation was performed using primary calf thyroid cells and a permanent cell line of IB-RS-2 cells; indirect sandwich ELISAs for FMDV, SVDV and VSV were used to characterize the specificity of the virus serotype in original material and cell culture antigens derived from them (Roeder and Le Blanc Smith, 1987; Ferris and Dawson, 1988; Ferris and Donaldson, 1988); in addition, real-time RT-PCR procedures (King et al., 2006; Shaw et al., 2007) were undertaken on samples received post-July 2002 (such assays had yet to be implemented and used routinely for diagnosis prior to this date).

2.5 Lateral flow device sample buffer and test operation

The results are based on the use of a proprietary buffer of Svanova Biotech AB. Aliquots of the epithelial suspensions were mixed with an equal volume of the LFD sample buffer and 200 µl of the mixture was applied to the sample pad of the LFD. If present in the sample, FMDV binds to the gold conjugate and forms an immune complex, which migrates by capillary action along the membrane until it reaches the immobilised antibody in the 'T' (test) window where it is trapped resulting in the accumulation of colloid gold, which can be visualised as a red line to signify a positive result. Excess (or unbound) Mab-labelled gold particles continue to migrate along the device until being captured by the immobilised rabbit anti-mouse antibody and the formation of a red 'C' (control) line, to validate the test. The test (and control) lines were observed for colour development at intervals over a period up to 1h from sample addition and reactions scored subjectively from

negative to strong. A diagrammatic representation of negative and positive LFD test development is illustrated in Fig. 1

2.6 Simple sample homogenizers

Two alternative devices for sample homogenization were evaluated for their potential abilities to prepare epithelial suspensions under field conditions for use in the LFD in comparison with the pestle and mortar that is employed routinely in the laboratory. These were (i) a disposable pellet pestle with microtube (Anachem) and (ii) a plastic rod (The BMC Research Workshop, Uppsala University, Uppsala, Sweden) in combination with a disposable glass bottle. Approximate 0.1 g amounts of similar pieces of each of 20 positive epithelia were ground in 1 ml volumes of the LFD sample buffer by each homogenizer. After leaving the homogenates for 2-3 min to allow the tissue to settle from the suspension, 200 µl of the supernatants were added to the LFD and reactions scored.

2.7 SAT 2 FMDV type-specific lateral flow device

Three SAT 2 FMDV type-specific Mabs (designations: 2H6, 3C5 and 4A6) produced at the IZS, Brescia against the virus strain ZIM 5/81 were selected for the production of prototype LFDs for SAT 2 FMDV antigen detection for preliminary evaluation (Grazioli et al., 2006).

3. RESULTS

3.1 Sensitivity and specificity of the 1F10 lateral flow device (LFD)

Table 1 shows the results observed with the 1F10 LFD in comparison with the antigen ELISA. Samples are sub-grouped into epithelia suspensions classified as either positive for one of the seven FMDV serotypes, negative (from naïve animals) or "NVD" (i.e. no virus detected by virus isolation/ELISA and/or RT-PCR). It can be seen that the overall specificity and sensitivity of the LFD is comparable to the ELISA with individual variations depending on the virus serotype – higher for FMDVs of serotypes A, C, SAT 1 and Asia 1, slightly lower for types O and SAT 3 but lower still for type SAT 2. Five epithelial suspensions containing high amounts of SVDV and VSV, respectively, (as judged by high OD values from homologous ELISAs) were all negative in the LFD.

3.2 Field sample preparation

Suspensions from 20 FMDV positive epithelia were prepared by each of the three sample extraction methods (i.e. [i] conventional pestle and mortar in comparison with [ii] the Anachem pellet/pestle with microtube and [iii] plastic rod with bottle) and tested in the 1F10 LFD. Positive reactions were produced in each case, although there were slight variations in the strength of test reactions: LFD reactions arising from the pellet/pestle and rod preparations were occasionally weaker than those from the pestle and mortar; three samples were slightly stronger by pellet/pestle than rod preparation and vice versa for three others (results not shown).

3.3 Prototype lateral flow devices for FMDV type SAT 2 detection

Thirty SAT 2 FMDV samples were tested in each of the three different SAT 2 Mab based prototype LFDs. All 30 reacted positively in the 2H6 and 4A6 devices, while 23 were also positive in the 3C5 LFD but with weaker reactions. For comparison, one of the 30 samples was negative by ELISA and only 19 had reacted positively in previous testing using the 1F10 LFD (and also to a weaker extent than now). Three type O, three A, two C, three SAT 1, two SAT 3, three Asia 1 and two SVDV positive samples, six negative and 18 NVD test samples failed to react in the 2H6 and 3C5 devices; conversely, some very weak non-specific reactions resulted in the 4A6 LFD.

4. DISCUSSION

Speed of diagnosis is paramount in maximizing the efficiency of the control measures that are implemented to stop the spread of disease and bring about its eradication when an outbreak of FMD occurs in a previously FMD-free country. Laboratory confirmation of the disease agent and its serotype are an essential component of this process, especially for primary outbreaks of the disease, where a battery of tests is often used to give the highest possible confidence in the results obtained. In secondary outbreaks where there may be less urgent need for characterization of the strain of virus involved, delays in sample shipment to the laboratory for confirmatory diagnosis can place reliance on clinical and epidemiological considerations for urgent decisions, such as whether or not to slaughter stock. For example, in the 2001 UK FMD outbreak, all susceptible livestock had to be slaughtered on infected premises (IPs) within 24 hours of disease confirmation and those on

dangerous contacts and contiguous premises within 48 hours (Anon, 2001); the consequence being that the majority of outbreaks were 'confirmed' on the basis of clinical judgment before laboratory results were known.
Unfortunately, clinical diagnosis of FMD can often be fraught and syndromes such as idiopathic mouth ulcers or ovine mouth and gum obscure disease (Watson, 2004), foot rot, trauma, grazing on rough pasture and agents other than FMD can be confused with it and can lead to the over-reporting of disease (Ferris et al., 2006). The availability of a specific, simple, cheap and disposable test that would be capable of providing results within minutes of taking a clinical sample and which could be used on a suspect premises by a veterinarian might help mitigate this. Accordingly, the development of rapid tests for FMD diagnosis was recommended by more than one of the committees of enquiry that investigated aspects of the 2001 UK FMD outbreak (Anderson, 2002; Royal Society, 2002)

The aim of this study was to develop a lateral flow device (LFD) that would meet this requirement and a pan-reactive FMDV Mab (1F10) was identified as the basis for its construction; thereafter a full laboratory validation study was undertaken.

It is evident from the validation that the specificity (99%) and overall sensitivity of the 1F10 LFD (83.6%) are almost identical to those of the slower and more complicated antigen ELISA (85%) for detection of FMDV antigen.

Although the LFD reacted with FMD viruses of all serotypes, it performed less well with FMDVs of the SAT 2 serotype and often weak reactions resulted, with several isolates not reacting at all. Steps are now being taken to address this possible shortcoming through the development and further validation of a SAT 2 type-specific LFD using the Mab 2H6; trials of prototype devices have produced encouraging results and suggest that the two devices would decrease the likelihood of SAT 2 FMDVs going unrecognized if used in concert.

In common with the antigen ELISA, the LFD is most suited for the detection of FMDV in vesicular fluid or epithelia but it is not recommended for investigating other sample types including blood, throat swab (probang) and milk samples, which would normally be expected to contain insufficient amounts of virus for recognition. For epithelia, the sample must first be homogenized to release virus antigen from the tissue. The evaluation of two simple sample preparation devices suggested that either could be used successfully for preparing such homogenates at the pen-side but that the rod/bijou device might be more practical and easier to manipulate. A pair of scissors and forceps would be additionally useful both for sample collection in the first place and then for finely mincing the specimen to facilitate grinding.

It is important to remember that unlike virus isolation or RT-PCR, the device does not have exquisite sensitivity for antigen detection (rather that it is equivalent to the antigen ELISA) and a negative result will not necessarily negate the possibility of an FMD infection. Nevertheless, the correct samples taken from animals in the acute stage of disease or soon after would normally be expected to contain sufficient amounts of virus to be detected by the 1F10 LFD. An additional advantage of its speed and potential ease of use in the field, is that if an unexpected result occurs it is very easy to repeat tests on the same or other affected animals. Experience from using the LFD in the UK 2007 outbreak also demonstrated that the LFD was extremely useful for confirmatory diagnosis in the laboratory because a result could be obtained within an hour of a samples arrival, compared to four hours for ELISA or RT-PCR.

The results of this study indicate that the 1F10 LFD has the potential for use in the pen-side next to an animal and for reassuring the veterinarian on the accuracy of his FMD diagnosis from clinical examination. Furthermore, the use of the LFD in FMD-endemic countries may also prove fruitful in, for example, overcoming problems associated with delays in submission of samples to reference laboratories, poor communication and under reporting of disease. The device is specific, sensitive, rapid to use and disposable. In order to incorporate use of FMDV detecting LFDs into procedures for the diagnosis of FMD, consideration will have to be given as to who should receive and use the devices and for the requirement for continued submission of samples to central laboratories for further characterization (e.g. sequencing) and for confirmation of LFD diagnoses.

5. ACKNOWLEDGEMENTS

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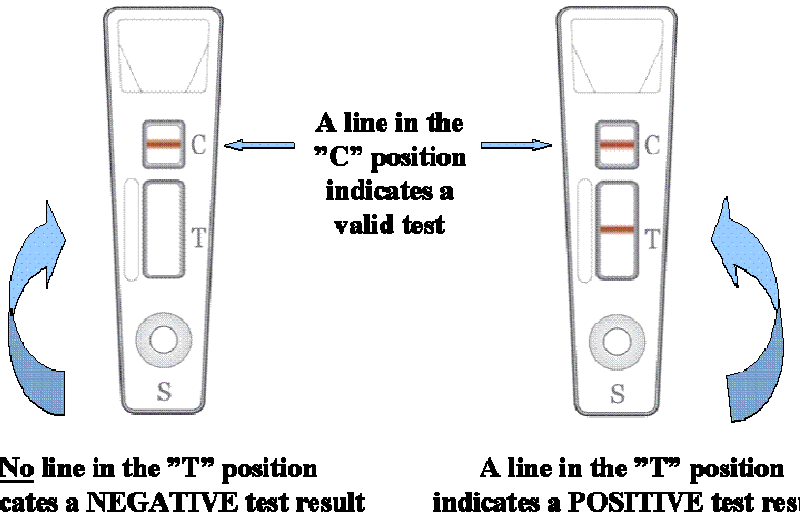

Fig.1: Diagrammatic depiction of negative and positive results produced in the 1F10 lateral flow device.

Table 1: Overall sensitivity and specificity of the 1F10 lateral flow device (LFD) using LFD sample buffers A and B with positive and negative epithelial suspensions in comparison with the FMDV antigen ELISA

Positive samples						
FMDV serotype	1F10 LFD			ELISA		
	No. tested	No. positive	Sensitivity (%)	No. tested	No. positive	Sensitivity (%)
O	131	121	92.4	128	122	95.3
A	41	36	87.8	41	32	78.0
C	24	15	62.5	24	14	58.3
SAT 1	24	16	66.7	24	13	54.2
SAT 2	34	20	58.8	34	30	88.2
SAT 3	10	7	70.0	10	9	90.0
Asia 1	40	39	97.5	40	36	90.0
Total	304	254	83.6	301	256	85.0
Negative samples						
Sample			Specificity (%)			Specificity (%)
SVDV[a]	5	0	100	5	0	100
VSV[b]	5	0	100	5	0	100
Negative[c]	7	0	100	7	0	100
NVD[d]	735	1	99.9	725	1	99.9
NVD[e]	251	4	98.4	251	0	100
Total	1003	5	99.5	993	1	99.9

[a] SVDV, swine vesicular disease virus epithelial suspensions positive by SVDV antigen ELISA
[b] VSV, vesicular stomatitis virus epithelial suspensions positive by VSV antigen ELISA
[c] negative epithelia from naïve animals
[d] NVD, no virus detected by virus isolation and antigen ELISA
[e] NVD, no virus detected by virus isolation, antigen ELISA or RT-PCR