OPTIONS FOR DECENTRALISED DIAGNOSIS OF SECONDARY CASES OF FOOT-AND-MOUTH DISEASE IN ANY FUTURE OUTBREAK.

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When foot-and-mouth disease (FMD) occurs in a country previously recognised as free from the disease, that country must report this to international agencies and trading partners with inevitable consequences – loss of disease-free status and disruption to export trade in livestock and animal products. Consequently, huge emphasis is placed on the accuracy of diagnosis of the first case of the disease in any outbreak and this diagnosis will always have to be confirmed by a national (or international) reference laboratory (NRL). Thereafter, the priority for the national veterinary authorities is to bring the disease under control as quickly as possible, to regain FMD-free status and to resume trade. Critical to FMD control is that as soon as the primary case has been diagnosed, all other secondary cases are rapidly identified so that appropriate measures can be taken to prevent further spread of the disease. However, confirming suspect cases at an NRL often involves an unavoidable delay in transporting specimens whilst the reference test methods employed by NRLs can be relatively slow and time-consuming. One approach to this problem is to establish rapid, high throughput methods at NRLs and to speed up the time taken to deliver samples. Alternatively, rapid test systems could be deployed outside of the NRL, making it feasible to move the confirmatory diagnostic process nearer to the location of affected animals in any future outbreak. This paper attempts to summarise options that are currently available for the direct diagnosis of secondary cases of FMD in an outbreak situation and to address some of the issues that arise if testing for FMD is to be performed outside of specialised high security laboratory facilities.

A confusing multiplicity of terminologies have been used to describe the concept of testing for FMD outside of an NRL; the terms “rapid testing”, “penside testing”, “on-farm testing”, “on-site testing”, “field testing”, “devolved testing” and “decentralised testing” have all been used synonymously. Assuming that NRLs are the centres designated for testing within their respective national territories, the term “decentralised testing” will be used throughout this paper to refer to all testing outside these laboratories, including that performed in regional laboratories.

Direct diagnosis of FMD – basic principles of currently available test methods

Direct diagnosis of FMD implies detection of the virus in specimens collected from suspect animals as distinct from indirect diagnosis, which is detection of an FMD specific antibody response.

Formerly, direct diagnosis was based on examination of tissue or vesicular fluid by a serotyping ELISA and/or isolation of FMD virus in tissue culture followed by use of a serotyping ELISA. The requirement to use virus isolation in cases where the ELISA on its own was negative or where the sample type was not appropriate for direct use of ELISA meant that testing could only be safely attempted in high security, specialised testing facilities (NRLs). Maximising the sensitivity of virus isolation, requires cultivation on calf thyroid cells (Snowdon, 1966). This is a primary cell line that cannot be maintained indefinitely in culture (and must be continuously harvested de novo), such that for practical reasons, many NRLs must rely on attempted isolation in less sensitive continuous cell-lines such as 1B-RS-2, lamb kidney and BHK cells. Although tissues containing large quantities of virus may give a positive test result within a few hours (if positive by ELISA without virus isolation), it takes 4 days (two passages of virus isolation of 48 hours duration each) to rule out the presence of infectivity in those specimens that test negative on virus isolation and antigen ELISA. For these various reasons alternative test methods, which detect either viral antigen or viral
genome and which give a positive or negative test result within a few hours, have been adopted by most NRLs.

**Detection of viral antigen (by antigen-antibody immunoassay)**

An antigen detection ELISA, developed at Pirbright (Roeder and LeBlanc Smith, 1987), has been used routinely for diagnosis of FMD (and swine vesicular disease; Ferris and Dawson, 1988) and for serotyping the virus for the past 20 years. This method, which does not involve an amplification step, requires relatively large amounts of the virus to be present in the tissue under test to yield a positive result.

A more recent development in FMD diagnostics has been modification of a technology (initially commercialised in human pregnancy diagnostic kits), which is based on the diffusion of coloured, antibody-coated latex beads or colloidal gold particles through a membrane towards an immobilising band of trapping antibody. These tests are referred to as either "rapid (immuno)- chromatographic strip tests" or "lateral flow devices" (LFDs). A prototype LFD has been shown to detect FMD virus with approximately equal sensitivity to the antigen capture ELISA (Reid et al., 2001) and performed satisfactorily with specimens from two suspected premises during the UK outbreak in 2001 (Ferris et al., 2001). An LFD produced by Svanova has been validated recently (Ferris et al, in press) and was used in the laboratory as an initial rapid test during the 2007 UK FMD outbreak besides being used successfully on one farm by a veterinarian to diagnose FMD during that epidemic (Ryan et al, 2008).

If FMD viral antigen is present in sufficient concentration in the test specimen, a visible line will appear in the "test window" of the device within minutes. The test is thus rapid and easy to perform. However, current formats of this test do not serotype the virus present, although this may be considered of lesser importance in the confirmation of secondary cases. Reading the device result by eye involves a degree of subjectivity; LFD readers are now available, but a requirement for their use could limit availability of the test and add to disease containment concerns. As the sensitivity of these devices is relatively low (as for the ELISA), they are only suitable for testing epithelial lesion material and vesicular fluid (which is expected to have the highest concentration of virus) and more than one affected animal within any particular group or herd of animals should be sampled and tested (on separate devices) to minimise the risk of a false negative test result. An advantage of their ease of use in the field is that where an unexpected result is obtained a repeat test could be done immediately, on the same or additional animals.

The initial step in testing epithelial lesion material by any antigen detection method requires disruption of the tissue to release viral antigen, a process performed in the laboratory by grinding the tissue with sterile sand, pestle and mortar. To facilitate field use of lateral flow devices, a simple dropper bottle containing sand and a buffer has been developed into which an epithelial specimen can be placed; after grinding with a pestle the supernatant can be added to the test device. Vesicular fluid, or a swab which has absorbed vesicular fluid, can also be added to the buffer prior to testing. The buffer fluid is then applied to the device.

**Detection of viral genome (by RT-PCR)**

FMDV-specific RNA (viral genome) may be amplified by the twin processes of reverse transcription or "RT", which produces a DNA copy of an RNA template, and the polymerase chain reaction or "PCR", which is capable of generating millions of copies of a target sequence of DNA. The amplified product may be detected by electrophoresis in a gel once the reaction is complete (in "conventional RT-PCR") or now more commonly and with greater sensitivity, specificity and speed in "real time" (i.e. as the amplification process is occurring) by using fluorescent probe technology.

There are four steps involved in the testing process: (i) extracting RNA from tissue specimens; (ii) the RT reaction; (iii) the PCR reaction and (iv) the detection of the amplified product.

The first of these steps is required to remove inhibitory substances that might be present in the sample. It is both time consuming and labour-intensive if done manually (imposing limits on both throughput and turnaround time) but has been greatly facilitated in recent years by the application of automated (robotic) extraction processes.

The PCR reaction itself requires very exacting conditions: a “cocktail” of enzymes, substrates and co-factors in very precise concentrations and repeated “thermal cycling” between two specific incubation temperatures (a higher temperature that allows double stranded DNA to separate and a
lower temperature that allows the resulting single strands to be replicated). Precise thermal cycling can only be achieved with precision instrumentation.

The final step in the process (detection of amplified product), if performed “in real-time”, requires sophisticated equipment and computer software.

Amplification processes such as RT-PCR are super-sensitive, such that cross-contamination is a particular concern and stringency is needed to generate reliable test results; in the testing laboratory this is usually achieved by complete separation of the various stages of the process, which are performed in separate rooms, thus avoiding amplified product from one test run contaminating test specimens prior to amplification in subsequent test runs. Centralised testing justifies the acquisition of sophisticated robotic equipment that enables large numbers of samples to be processed at once with a high degree of fidelity.

Several different companies have developed portable “real-time” PCR equipment in recent years. These platforms are robust and easy to use, having been designed for use by the military (to detect infectious agents such as Bacillus anthracis, spurred on by fears of bio-terrorism). Combined with mobile robotic RNA extraction equipment (or simple mechanical shaking devices), this machinery is currently being evaluated for decentralised diagnosis of FMD (King et al., 2008). Pre-packaged PCR reagents, validated for FMD virus detection, are also commercially available and it will be possible to perform the entire RT-PCR process within disposable sealed tubes considerably reducing the risk of cross-contamination, although most systems currently on the market do not perform the nucleic acid extraction step. With this type of equipment, only small numbers of samples (1-8) can be processed concomitantly.

Alternative methods of nucleic acid amplification have been developed which are isothermal (i.e. do not require thermal cycling and such precise instruments) and some of these processes can be formatted to produce a visible colour change if positive, allowing the test to be read without sophisticated equipment (Dukes et al., 2006; Lau et al., 2008). This could form the basis of a disposable decentralised testing kit; but none of these methods are yet routinely applied in FMD diagnosis.

Current genome detection methods are not typically designed to serotype FMD viruses. Use of a combined testing strategy involving both rapid antigen detection and rapid genome detection provides separate lines of evidence for the presence of infection and hence greater confidence in the diagnosis. However, in practice, this may not always be feasible (e.g. samples such as blood cannot be tested directly by antigen ELISA) and judgement must be used as to the level of certainty required from laboratory confirmation, according to the strength of the available field evidence (i.e. the clinical basis for suspecting FMD) and the consequences of the diagnosis.

Currently available rapid testing options for diagnosis of secondary cases

**OPTION 1**

Retain all testing for FMD at the NRL; take steps to expedite transfer of samples to the laboratory and use a combination of LFD, antigen detection ELISA and RT-PCR testing. The advantages are obvious in that the testing is performed within specialised (QA-accredited) bio-secure facilities by skilled staff experienced in performance characteristics of the test methods and in the interpretation of the test results. A high throughput of samples is possible (such as that required for active surveillance programmes). The principal disadvantages are the delay in getting specimens to the laboratory and in completing the testing process; with the current methodology, it would take about five hours from receipt of the specimen in the NRL until testing is completed by all three procedures and this presumes that there are no discrepant or inconclusive results requiring a retest (but LFDs can be used in NRLs to give very rapid preliminary results as done in the UK in 2007)

**OPTION 2**

Perform real-time RT-PCR in a local, regional or mobile laboratory using RNA extraction and PCR equipment or using a modularised portable PCR unit (such as the Bioseek Vet that is in development, Smiths), testing samples that have been inactivated by agents such as TRIzol. Local laboratory staff could perform the test and a test result could be available within two hours from receipt of the specimen in the laboratory. It should be noted that the EU Community Reference Laboratory (CRL) see few advantages in using mobile laboratories over local/regional laboratories,
where these are available; for example in the UK, where the proximity of most farms to a regional laboratory means less than 1.5 hours driving distance. An important caveat is that whilst it would be feasible to set up local laboratories in advance or at short notice with simple automated equipment to enable rapid testing of a low throughput of samples, this would not give a high throughput service (as available at the NRL) for testing large numbers of samples at the same time, in the event of a major local outbreak. However, some regional laboratories may already have capacity for this type of testing for endemic diseases.

**OPTION 3A**

Use LFDs on the suspect premises. The official veterinarian that will have examined and sampled the suspect animals could perform this method and a test result could be available within 10-15 minutes providing that a moderate concentration of antigen is present in the test sample. The low cost of the individual LFDs would facilitate their local distribution in “peacetime”, thus ensuring that devices would be quickly available for suspect cases. They could, for instance, be routinely included in the FMD field investigation kit. The official veterinarian would therefore have them immediately available while on the suspect farm.

**OPTION 3B**

Use a modularised portable PCR unit (e.g. Bioseeq Vet, Smiths) on the suspect premises. Following notification of a suspect case, this would require transport of the device to the farm, where it could be used to detect viral genome in blood, epithelium, OP fluid or other samples. A result would be available 60-90 minutes later. The advantage of this approach is that it would be more sensitive than the LFD, while still enabling on-farm testing. A disadvantage is that, due to the cost of these devices, they are likely to be stored in some regional depot rather than one being issued to each official vet. Therefore the device would have to be transported to the suspect farm following a request by the investigating vet. This time delay may be insignificant in some circumstances, but in other instances it may be just as quick to simply transport the samples to a regional or local laboratory (as discussed in option 2 above).

There is a trade-off or compromise between proximity of the testing process to the affected animal (allowing for speed of diagnosis) and performing the test in ideal laboratory conditions (ensuring reliability or accuracy of diagnosis). This compromise is summarised in Table 1. Of course, there is nothing to stop the national veterinary authority from applying these various options in series until satisfied that there is sufficient evidence to deem a herd to be infected or free from infection. Such a hierarchy of testing options is illustrated diagrammatically in Figure 1. One crucial point in support of this scheme, which may not be readily apparent from Figure 1, is that the animals which pose greatest risk (because they are likely to be shedding the most virus) would most probably be rapidly identified as positive using on-site tests (Options 3a or 3b) whilst the infected animals that pose least risk (as they are shedding the least amount of virus) should eventually be identified as positive by the combination of more sensitive test methods which are employed at NRLs (Option 1). In other words the infected animals that pose the greatest risk should be identified fastest.

An issue that is sometimes overlooked is that if samples are not submitted to a NRL they may not become available for subsequent analysis; for example for genome sequencing to trace routes of transmission. A possible way of overcoming this problem would be to ensure that either epithelial suspensions prepared in the field for use in LFDs (or other detection systems) or duplicate vesicular epithelium are sent on to the NRL immediately afterwards.

Alternatively, options 1, 2 and 3a might be conducted in parallel and would probably provide the optimum balance of speed and reliability.
### Table 1: Relative ranking of different options for diagnosis of secondary cases of FMD during an outbreak (1 = best, adapted from Reid et al., 2006)

<table>
<thead>
<tr>
<th>Option</th>
<th>Location</th>
<th>Method</th>
<th>Relative ranking of testing process</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Speed</td>
</tr>
<tr>
<td>1</td>
<td>NRL</td>
<td>AgELISA and/or rtRT-PCR (Delivery + 5 hrs)</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Regional or mobile lab.</td>
<td>rtRT-PCR (Delivery + 1.5 - 5 hrs)*</td>
<td>2</td>
</tr>
<tr>
<td>3a</td>
<td>Suspect premises</td>
<td>LFD (15 minutes)</td>
<td>1</td>
</tr>
<tr>
<td>3b</td>
<td>&quot;</td>
<td>Portable rtRT-PCR (1.5 hrs)</td>
<td>1/2</td>
</tr>
</tbody>
</table>

§Assumes conventional testing methods but LFDs and/or a bioseq can also be used at the NRL for occasional (i.e. low throughput) high speed testing of urgent samples; *Depends on the method used

### Issues that will arise with decentralised testing for FMD

The veterinary authority in each country must decide which of the currently-available options would be most appropriate to their needs in the event of any future outbreak. Some national authorities have already given considerable thought to how they might decentralise testing for FMD (and other OIE-listed diseases) in the event of a future disease outbreak; some examples are summarised at the end of this paper (Annex 1). If a decision is taken to opt for decentralised testing, a number of predictable issues arise; these issues are listed and briefly outlined below. Each of these issues could and should be resolved in "peacetime”. Action points are suggested after each item.

1. **Comparative evaluation and field validation of rapid test systems.**

   Several studies have been conducted in which one or other of the rapid test systems described above have been compared with conventional laboratory-based testing methods for FMD (Reid et al., 2001; Hearps et al., 2001; Callahan et al., 2001, Ferris et al, in press), demonstrating the efficacy of these methods. However, to the authors’ knowledge, none of the currently available test systems have been fully validated for field use. The authors recommend that the three testing options described above should be applied in parallel and their performance compared in a real "outbreak” situation. A potential opportunity for such field evaluation and validation exists in Anatolia, where FMD is endemic and where such a study could compliment other work already in progress in that region to better understand and control the spread of FMD. An LFD supplied by Princeton Biomedical Corporation was employed with limited success during a pilot study on FMD outbreak investigation conducted in Erzurum in 2004 (Bulut et al., 2004). Planning is currently underway to carry out the initial phase of a field trial using both the 1F10 LFD (Svanova) and the Bioseq Vet (Smiths) in Turkey, most likely in Erzurum.

   **Action:** Recommend continuing support to representatives of the Şap institute (Ankara), the Turkish veterinary authorities, the EUFMD Research Group, and the CRL in their efforts to carry out this study in late 2008 and early 2009.

   It is important to note that following a primary diagnosis of FMD in any future outbreak, the NRL must ensure that whatever test system will be applied for diagnosis of secondary cases is capable of recognising the causative strain of the virus.

2. **Availability of test devices, reagents and equipment**

   Options 3a and 3b require that sufficient LFDs or Bioseq devices are purchased and stored or that a contract for supply is agreed with a commercial source, in advance of an outbreak. Both the shelf-life and cost of devices should be considered when making these arrangements. Option 2
requires that regional or mobile laboratories are equipped with the necessary precision instrumentation; the machinery could be provided in advance (and possibly put to other uses in peacetime) or instruments could be stored at the NRL and delivered to where they are most urgently required during an outbreak (the machines could be relocated during the outbreak as events dictate). With respect to either testing option, commercial companies need incentives to develop, validate, produce and market FMD testing systems during peacetime if such tests are to be commercially available when required during an outbreak.

**Action:** representatives of the national veterinary authority should decide in consultation with colleagues in the NRL; EU member states should consult with DG-SANCO on the possibility of LFDs and/or portable PCR units being included in an EU diagnostic reagents bank; the concept of a reagents bank has been the subject of a previous EUFMD position paper by Haas (2003).

3. **Determine the levels of proof required to cull a herd or derestrict a herd**

Who will decide to cull or derestrict a herd? On what basis will this decision be made? Thought should be given as to how decisions will be made at a herd level, and how test results and other evidence (epidemiological and clinical information) will be considered in the decision-making process. It is critical that this is discussed and agreed in advance of an outbreak whilst there is time available for debate.

**Action:** representatives of the national veterinary authority should discuss with colleagues in the NRL and should attempt to formulate a “decision tree”.

4. **Training and/or instruction in the use of rapid test systems**

Options 3a and 3b require that clearly-written, step-by-step instructions are provided with the test devices, such as those developed by FAO for the use of LFDs in the diagnosis of rinderpest (Roeder, 2002). Option 2 will require staff training.

**Action:** the NRL (and perhaps the CRL) could provide instructions and training as necessary.

5. **Biosecurity risks and their mitigation**

Option 3 above does not pose a biosecurity risk (over and above that associated with any field investigation of suspect cases) as the testing is done on the suspect premises and the LFD devices may be discarded or sent to the NRL once the test has been performed. The Bioseq Vet device may be completely submerged in disinfectant, and the reaction cartridges (containing inactivated samples) may be discarded or sent to the NRL once the test has been performed.

Option 2: to avoid the prospect of live FMD virus being handled in non-biosecure laboratory facilities, samples could be treated on collection so as to destroy infectivity whilst preserving RNA. However, this may limit the scope for subsequent characterisation of virus in samples scored as positive.

As regards option 1, all NRLs for FMD should operate to the prescribed standards (Anon., 1993).

**Action:** for option 2, protocols for treatment of samples should be developed and agreed that will ensure loss of infectivity without affecting viral RNA recovery.

Rapid testing systems (for FMD diagnosis) could potentially be used in peacetime to give an early indication of infection when investigating suspected cases of FMD. They could also be used in active surveillance for FMD in animals that have been sampled and are being tested for the presence of other infectious agents. However rapid test systems (or “penside tests”) are not the panacea for FMD diagnosis and control that some critics of UK government policy in 2001 would contend. Early recognition of the disease will still largely depend on the awareness, vigilance and goodwill of the farming community and practising veterinarians. A rapid and proportionate response to suspected cases will require that national veterinary services are staffed by competent experienced veterinarians who can recognise the clinical disease and are capable of rational decision-making. Furthermore, regardless of what test systems are deployed in any future outbreak, the services of a national reference laboratory will be indispensable, not only to confirm the primary case but to oversee all subsequent testing for the presence of the disease and to co-ordinate if not prosecute the large-scale serological testing required to substantiate freedom from FMD after an outbreak.

**REFERENCES**
ANNEX 1

DECENTRALISED FMD TESTING IN FRANCE

A network of five regional laboratories has been put in place in order to perform FMD serology in the event of a future outbreak. These five laboratories have to participate in an annual ring test organized by the national reference laboratory (NRL), AFSSA, Maisons-Alfort. Moreover, they must test 800 sera per year in order to maintain competence. A similar network of five regional laboratories (each with high containment facilities) is to be put in place for rtRT-PCR testing to
detect FMD virus. As for serological testing, these laboratories would have to participate in ring-tests organized by the NRL; in addition regional laboratory staff would receive training from the NRL in biosafety measures that have to be followed. Suspected cases of FMD would continue to be sent to the NRL. The regional laboratories would only be authorised to perform rtRT-PCR in the event of FMD being confirmed by the NRL and only in the case of suspected secondary cases. Some of the laboratories have systems in place for collection of samples on farm. Alternatively, in the case of remote farm locations, a transport system will be put in place to ensure biosecure delivery of specimens to the nearest laboratory. Testing would be performed under the supervision of the NRL and all results would be sent electronically to the NRL for interpretation; no result would be released without NRL authorisation. The extent to which such a system would be used in any future outbreak is difficult to gauge and will depend on such factors as the number of outbreaks and the outbreak locations. Decentralised rtRT-PCR testing is already in place for both bluetongue and avian influenza and has been extensively used for both; a network of ISO-accredited laboratories has been operating under control of the relevant NRL. The requirement for NRL oversight to ensure confidence in test results, imposes an upper limit on the number of laboratories that could be included in such a network.

DECENTRALISED FMD TESTING IN GERMANY

There are three strands to either current contingency plans or applied research for decentralised FMD testing in Germany: (i) in case of an FMD epidemic - serological testing (by NSP ELISA) in laboratories of the German States (“Länder”) using reagents from a national test kit bank and testing sera derived from low risk premises in which no clinical signs of FMD have been seen; (ii) in “normal times” - screening of samples (by qRT-PCR at German State laboratories) to rule out FMD as a potential, but unlikely differential diagnosis, e.g. in suspected cases of bluetongue. (Note: Where there is a clinical suspicion of FMD, specimens would be delivered with minimum delay by special courier, e.g. police helicopter, to the national reference laboratory FLI, Insel Riems) and (iii) a research project involving the validation of portable PCR machines that might eventually be deployed for use in the field by special task forces with clinical specimens derived from experimentally-infected animals.

DECENTRALISED FMD TESTING IN ISRAEL

In Israel FMD control policy consists of annual vaccination (with 150% coverage of the national herd) and quarantine in the event of an outbreak. The main problem in dealing with new outbreaks of FMD is the time interval that elapses between first appearance of clinical signs and first recognition of the disease. Rapid test methods will not necessarily ensure rapid recognition of FMD. Of much greater importance in this regard is that all persons involved with the livestock industry are aware of the disease and remain vigilant (regular training to remind key persons about FMD may be very useful in this regard). An LFD (Svanova), will be distributed this year to all regional veterinary services stations (six throughout the country) and this device will be used to support the local veterinary officer when faced with a clinical suspicion of FMD, in deciding whether or not to place the affected herd under quarantine. This new approach will be used in addition to sending samples to NRL for confirmation by conventional testing methods; the time taken to reach the NRL with samples from anywhere in the country would not exceed four hours.