Appendix 45

Diagnosis of FMD by RT-PCR: prospects for mobile and portable assays

Scott M. Reid[*]*, Juliet P. Dukes, Katja Ebert, Nigel P. Ferris and Donald P. King*

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

Abstract:

Rapid and accurate diagnosis is needed for effective control of foot-and-mouth disease (FMD). Laboratory-based methods such as virus isolation, antigen-ELISA and real-time RT-PCR (rRT-PCR) can provide an objective result within a few hours of sample receipt. However, the time taken to transport suspect material to a centralized laboratory can be unacceptably long, often precluding laboratory confirmation in the event of an outbreak. There are opportunities to deploy mobile rRT-PCR assays inside a vehicle, or in local laboratories, for rapid diagnosis of suspect cases. These assays typically require pre-processing steps of samples (such as RNA extraction) which demands trained personnel. Portable PCR technology has also recently been developed with the potential for pen-side or "point of care" diagnosis. These field-based assays could be operated by untrained personnel without prior knowledge of molecular biology. In contrast to laboratory-based assays, where high sample throughput is a major prerogative, portable assays focus on speed in order to generate a diagnostic result within one hour of sample collection. To determine whether mobile and portable platforms might be used to carry out molecular diagnosis closer to suspect cases of FMD, two instruments were evaluated: (i) a "mobile" nucleic acid extraction robot (BioRobot EZ1, Qiagen) was compared with centralized robotic extraction equipment currently used to prepare RNA for FMD virus (FMDV) detection and (ii) the portable Bioseeq^{TM} platform (Smiths Detection) was assessed as to whether this instrument could be used for pen-side diagnosis of FMD by rRT-PCR. Both instruments provide realistic options for performing molecular assays for FMDV away from centralised laboratories.

Introduction:

Rapid and accurate diagnosis is needed for effective control and eradication of FMD. Laboratory-based methods such as virus isolation (VI), antigen detection ELISA (Ferris and Dawson, 1988) and real-time RT-PCR (rRT-PCR) (Reid et al., 2003; Shaw et al., 2004) can provide an objective result within a few hours of sample receipt. However, the time taken to transport suspect material to a centralized laboratory can be unacceptably long, often precluding laboratory confirmation in the event of an outbreak. Using existing available equipment, there are opportunities to deploy mobile rRT-PCR assays inside a vehicle, or in local laboratories, for rapid diagnosis of suspect cases (Callahan et al., 2002; Hearps et al., 2002). These assays typically require pre-processing of samples (such as RNA extraction) which demands trained personnel. Portable PCR technology has also recently been developed with the potential for pen-side or "point of care" diagnosis. Field-based assays like these could be operated by untrained personnel without prior knowledge of molecular biology. In contrast to laboratory-based assays, where high sample throughput is a major prerogative, portable assays focus on speed in order to generate a diagnostic result within one hour of sample collection. To determine whether mobile and portable platforms might be used to carry out molecular diagnosis closer to suspect cases of FMD, two instruments were evaluated. Firstly, the performance of a "mobile" nucleic acid extraction robot (BioRobot EZ1, Qiagen) was compared with centralized robotic extraction equipment currently used to prepare RNA for FMDV detection. Secondly, the portable Bioseeq^{TM} platform (Smiths Detection, Watford, UK) was assessed as to whether this instrument could be used for pen-side diagnosis of FMD by rRT-PCR.

Materials and methods:

<u>Comparison of the BioRobot EZ1 RNA extraction instrument with other extraction methods for diagnosis of FMD</u>

An initial evaluation of the Qiagen BioRobot EZ1 was performed by comparing RNA extracted by this potential mobile device with other robotic instruments, namely the Qiagen BioRobot 9604 and the MagNA Pure LC (Roche) (Fig. 1). The QIAamp Viral RNA Mini Kit (Qiagen) was also included to compare the performance of a manual extraction method. Decimal dilutions of an ~10% epithelial suspension of the reference FMDV strain O_1 Manisa (TUR 8/69) were prepared in suspensions of uninfected negative bovine epithelium and 200µl of each dilution was lysed in 300µl of the accompanying lysis buffer as appropriate (Table 1). For the manual RNA extractions, 140µl of the dilutions were lysed in 560µl of AVL

buffer (Qiagen). Each dilution series was extracted in triplicate for all RNA extraction systems, except for the BioRobot EZ1, where samples were processed in duplicates. Uninfected bovine epithelium was used as a negative control for each dilution series.

A **B** **C**

Fig. 1. Automated Extraction Robots. A) Qiagen BioRobot EZ1, B) Qiagen BioRobot 9604, C) MagNA Pure LC (Roche).

The initial sample volumes and final elution volumes for each extraction method were selected according to the respective pre-programmed purification protocols (Table 1).

Table 1 Comparison of the BioRobot EZ1 RNA instrument with other extraction methods

Extraction method/robot	Initial sample volume	Elution volume	Extraction time	Lysis buffer
BioRobot EZ1	400µl	75µl	~35 min	AVL Buffer
BioRobot 9604	500µl	50µl	~2 h	AVL Buffer
QIAamp Viral RNA Kit	700µl	60µl	~30-45 min	AVL Buffer
MagNA Pure LC	500µl	50µl	~1h 30 min	Lysis/Binding Buffer

A two-step rRT-PCR, similar to that previously described (King et al., 2006) was carried out on all extractions. In addition, RNA extractions prepared by the MagNA Pure LC and BioRobot EZ1 were amplified using the Superscript III Platinum® One-Step Quantitative RT-PCR system (Invitrogen, Paisley, UK), the diagnostic real-time assay currently used at the World Reference Laboratory for Foot-and-Mouth Disease, Pirbright. The PCR primers and probe targeted a conserved region within the internal ribosomal entry site (IRES) of the FMDV genome and have already undergone substantial evaluation (Reid et al., 2003; Boyle et al., 2004; Shaw et al., 2004; King et al., 2006; Ferris et al., 2006).

Evaluation of the Bioseeq™ portable rRT-PCR assay platform:
A rRT-PCR assay using the primers/probe set against the IRES was developed for the detection of FMDV using the Bioseeq™ device. This is a hand-held rRT-PCR instrument weighing approximately 3 kg with six independently programmable optical modules for real-time thermocycling. A decimal dilution series was prepared by titrating an ~10% epithelial suspension collected from a cow experimentally infected with O_1 Manisa into a negative bovine epithelial suspension. RNA samples prepared using the MagNA Pure LC as described previously (Reid et al., 2003), were amplified using the Superscript III Platinum® One-Step Quantitative RT-PCR system.
To show that original clinical samples could be tested using the Bioseeq™ without RNA extraction, vesicular epithelium from a cow infected with the O_1 Manisa isolate was placed into a disposable Nalgene® bottle containing 10 ml of phosphate buffered saline solution and six ball-bearings. The bottle was shaken for 3 minutes, after which 1 µl of the resulting suspension was tested directly by rRT-PCR in the instrument.

Results:

BioRobot EZ1 RNA extraction instrument:
Of the RNA extraction methods involving Qiagen robots/kits, the BioRobot EZ1 extractions produced lower C_t values for corresponding samples, which indicated a more efficient RNA isolation (Fig. 2). RT-PCR assays of the MagNA Pure LC extractions had a similar sensitivity and limit of detection to those

resulting from the BioRobot EZ1 extractions (Fig. 3). All extraction methods had a similar limit of detection with 10^{-5} being the highest dilution of the O_1 Manisa isolate that was consistently detected.

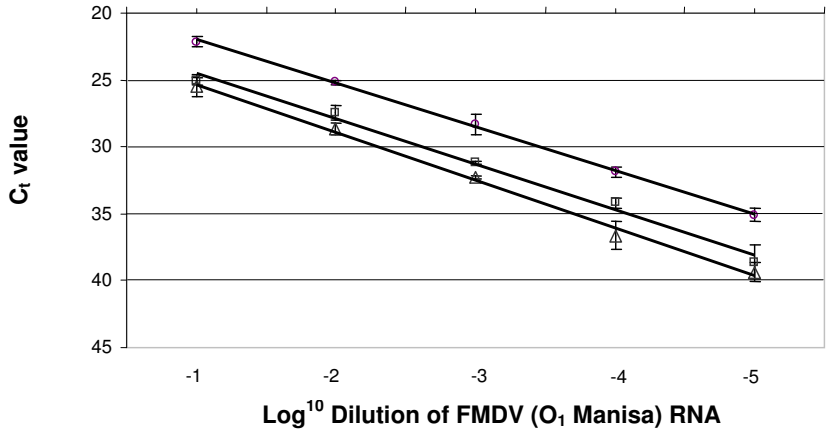

Fig. 2. Comparative detection of RT-PCR for FMDV using two-step rRT-PCR: (○) Qiagen BioRobot EZ1, (□) Qiagen BioRobot 9604, (Δ) QIAamp Viral RNA Mini Kit. Error bars represent standard deviation around the mean value.

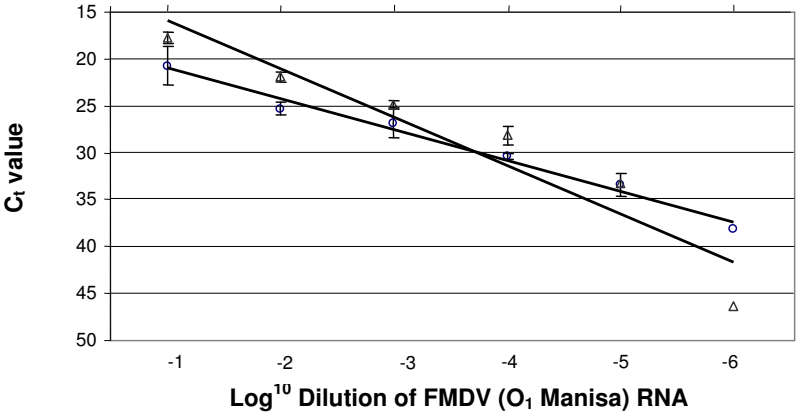

Fig. 3. Comparative detection of FMDV using one-step rRT-PCR: (Δ) Qiagen BioRobot EZ1, (○) MagNA Pure LC system.

Bioseeq™ portable rRT-PCR assay platform:

The Bioseeq™ can only test six samples at a time so that evaluation of this platform against large numbers of isolates was not feasible. However, limited testing of representative strains of all seven serotypes of FMDV indicated that the assay had similar diagnostic sensitivity to the laboratory-based assay previously described (Reid et al., 2003). Fig. 4 shows that the limit of detection using parallel samples was similar for both assays: the 10^{-6} dilution of O_1 Manisa RNA was the highest dilution that was consistently detected by either of the rRT-PCR assay platforms. These preliminary results indicate that it is possible to develop a sensitive rRT-PCR assay on the Bioseeq™ platform. Furthermore, a result could be generated directly from clinical material without nucleic acid extraction (data not shown).

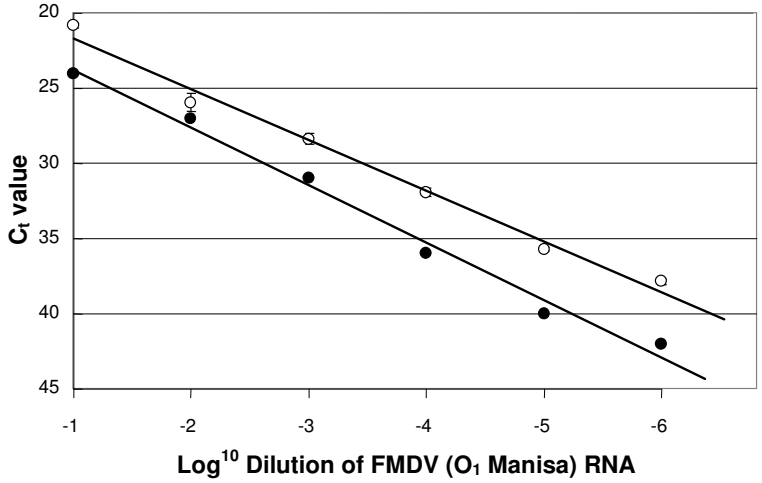

Fig. 4. Comparative analytical sensitivity of rRT-PCR assay for detection of FMDV on the Bioseeq™ (●) and a laboratory-based diagnostic real-time assay (○ Mx4000, Stratagene: Reid et al., 2003).

Discussion:

Validation of rRT-PCR assays in centralised laboratories such as IAH Pirbright has shown that these assays have high sensitivity which is equal to, or greater than, that of VI (considered to be the gold standard test for FMD diagnosis). These assays have significant practical advantages, which can be accelerated further by coupling them with automated liquid handling equipment for nucleic acid extraction and set up of the RT and PCR reactions for enhanced sample throughput. However, significant improvements to the turn-around time for samples cannot be made unless the time taken to transport them to the central laboratory for testing is drastically reduced. Implementation of the control strategy adopted by the UK government during the 2001 FMD epidemic: to slaughter the animals on infected premises with 24 hours of clinical diagnosis and those on neighbouring premises within 48 hours meant that the reported clinical signs were not confirmed by laboratory investigation. Retrospective analyses have indicated that reliance upon clinical diagnosis alone resulted in over-reporting of FMD, since the presence of FMDV on 23% of farms could not be corroborated by laboratory methods (Ferris et al., 2006). Development of rapid and sensitive technologies that could be deployed *in situ* without transferring the samples to a centralised laboratory might therefore be an option for improving the speed and reliability of FMD diagnosis.

Deployment of rRT-PCR to regional laboratories could speed up the time interval between sample collection and issue of assay result. Existing laboratory-based RT-PCR assays for FMDV could realistically be transferred out of the centralised laboratory to be performed at other laboratories. These tests would be operated by trained personnel in a "controlled" environment. However, this could cause logistic problems as virus containment must be adequate at these sites. This could be addressed by inactivating samples prior to receipt since RT-PCR does not depend upon the presence of live virus but would prevent confirmatory testing of the samples by VI.

This study showed that RNA extractions performed using the BioRobot EZ1 can accelerate the RT-PCR assay to produce test results within shorter time-scales and the comparatively light weight, size and user friendliness of this robot should make it highly suitable for use in regional and mobile laboratories for FMD diagnosis. On farm/farm gate diagnosis may be achieved using portable equipment such as the Bioseeq™ instrument. Both instruments provide realistic options for performing molecular assays for FMDV away from centralized laboratories.

Further validation of these and other mobile and portable instruments is required before RT-PCR methodology can reliably be used in the field or closer to suspect premises for FMD outbreak diagnosis and control. Together with the investigation of isothermal techniques (also presented in the meeting: Dukes et al., 2006), this equipment can potentially form part of the contingency planning towards combating future outbreaks of FMD.

Acknowledgements:

This work was funded by the Department for Environment, Food and Rural Affairs, UK (Project number SE1121) and the European Union Project LAB-ON-SITE (SSPE-CT-2004-513 645). The authors thank Qiagen for providing the BioRobot EZ1; Drs Ian Barker and Neil Boonham (Central Science Laboratory, York, UK) for sharing details of the ball-bearing method used for crude sample preparation in association with the Bioseeq™ and Smiths Detection both for supplying the Bioseeq™ and for invaluable technical assistance.

References:

Boyle, D. B., Taylor, T. and Cardoso, M. (2004). Implementation in Australia of molecular diagnostic techniques for the rapid detection of foot and mouth disease virus. *Australian Veterinary Journal*, 82, 421-425.

Callahan, J. D., Brown, F., Osorio, F. A., Sur, J. H., Kramer, E., Long, G. W., Lubroth, J., Ellis, S. J., Shoulars, K. S., Gaffney, K. L., Rock, D. L. and Nelson, W. M. (2002). Use of a portable real-time reverse transcriptase-polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. *Journal of the American Veterinary Medical Association*, 220, 1636-1642.

Dukes, J. P., King, D. P. and Alexandersen, S. (2006). Novel reverse transcription loop-mediated isothermal amplification for rapid detection of foot-and-mouth disease virus. *Archives of Virology*, 151, 1093-1106.

Ferris, N. P. and Dawson, M. (1988). Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular diseases. *Veterinary Microbiology*, 16, 201-209.

Ferris, N. P., King, D. P., Reid, S. M., Shaw, A. E. and Hutchings, G. H. (2006). Comparisons of original laboratory results and retrospective analysis by real-time reverse transcriptase-PCR of virological samples collected from confirmed cases of foot-and-mouth disease in the UK in 2001. *Veterinary Record*, 159, 373-378.

Hearps, A., Zhang, Z. and Alexandersen, S. (2002). Evaluation of the portable Cepheid SmartCycler real-time PCR machine for the rapid diagnosis of foot-and-mouth disease. *Veterinary Record*, 150, 625-628.

King, D. P., Ferris, N. P., Shaw, A. E., Reid, S. M., Hutchings, G. H., Giuffe, A. C., Robida, J. M., Callahan, J. D., Nelson, W. M. and Beckham, T. R. (2006). Detection of foot-and-mouth disease virus: comparative diagnostic sensitivity of two independent real-time RT-PCR assays. *Journal of Veterinary Diagnostic Investigation*, 18, 92-96.

Reid, S. M., Grierson, S. S., Ferris, N. P., Hutchings, G. H. and Alexandersen, S. (2003). Evaluation of automated RT-PCR to accelerate the laboratory diagnosis of foot-and-mouth disease virus. *Journal of Virological Methods*, 107, 129-139.

Shaw, A. E., Reid, S. M., King, D. P., Hutchings, G. H. and Ferris, N. P. (2004). Enhanced laboratory diagnosis of foot and mouth disease by real-time polymerase chainreaction. *Revue Scientifique et Technique Office International des Épizooties*,23, 1003-1009.