Use of automated RT-PCR to detect FMDV in milk

Scott M. Reid1 *, Satya Parida1, Donald P. King1, Geoffrey H. Hutchings1, Andrew E. Shaw1, Nigel P. Ferris1, Zhidong Zhang1, J. Eric Hillerton2 and David J. Paton1

1Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Woking, Surrey, GU24 0NF, UK
2Institute for Animal Health, Compton Laboratory, Newbury, Berkshire, RG20 7NN, United Kingdom

Abstract:
Introduction: Foot-and-mouth disease virus (FMDV) excreted in milk can play a role in the transmission of FMD. Using samples collected from experimentally infected cattle, the aim of this study was to assess the performance of automated RT-PCR as a diagnostic tool for the detection of FMDV in milk. Materials and Methods: Fore- and machine milk were collected over 4 weeks from 2 dairy cows inoculated with FMDV O UKG 34/2001, and from 2 in-contact cows. Clinical signs were closely monitored and correlated to the presence of FMDV in serum, "probangs", nasal and saliva swab samples. The whole, skim, cellular debris and cream components of the milks were tested by automated real-time RT-PCR and virus isolation (VI). Additional experiments investigating the effects of temperature stability and preservative treatment further evaluated RT-PCR for routine diagnosis of FMD in milk samples that might be submitted to the WRL. Results: The onset of severe clinical signs of FMD in all 4 cattle correlated with high levels of viraemia in the serum, and presence of FMDV in "probangs", saliva and nasal swabs. The RT-PCR results matched closely with those for VI in detecting FMDV in all milk components and generally coincided with, but did not precede, the onset of the clinical signs. RT-PCR was able to detect FMDV in milk up to day 23 post infection. The detection limit of FMDV in milk was greater by RT-PCR than VI. Furthermore, in contrast to VI, RT-PCR detected virus genome following heat treatment that mimicked pasteurisation. RT-PCR also detected FMDV in preservative (Bronopol/Kathon) treated milk. Discussion: This study shows that automated RT-PCR could be used for laboratory detection of FMDV in milk. The ability of RT-PCR to screen bulk milk tank samples and/or dairy herds may play a role in the control of FMD.

Introduction:
Foot-and-mouth disease (FMD) is characterised in dairy cows by fever, vesicular lesions, pyrexia and a reduction in milk yield. FMD virus (FMDV) excreted in milk from infected animals is thought to have played a role in the transmission of FMD in previous outbreaks (Dawson, 1970; Donaldson, 1997). Milk represents an ideal medium for laboratory diagnosis of FMD that may be particularly appropriate for the surveillance of disease in dairy herds. Previous studies have also demonstrated that the FMD virolactia can precede the presentation of clinical signs in experimentally infected animals (Burrows, 1968; Blackwell and Hyde, 1976; Blackwell et al., 1982) so that an assay detecting the presence of FMDV could potentially be used as a preclinical diagnostic tool.

Of the established diagnostic methods for FMDV, only virus isolation (VI) can be used to detect FMDV in milk samples successfully (Blackwell et al., 1982). The antigen-detection ELISA (Ferris and Dawson, 1988) currently used at the OIE/FAO World Reference Laboratory for FMD, Pirbright, is unsuitable for testing milk. Previous studies have demonstrated that automated RT-PCR is a valuable diagnostic procedure for the laboratory detection of FMDV in vesicular epithelial tissue (Reid et al. 2003; Shaw et al., 2004) and in serum, nasal swabs and oesophageal-pharyngeal scrapings ("probangs"; Zhang and Alexandersen, 2003). The aim of this study was to evaluate the performance of RT-PCR methods for the detection of FMDV in milk.

Materials and Methods:
Experimental infection and monitoring of cows with FMDV
Two adult milking cows held in separate pens were inoculated (day 0) by intra-dermolingual injection with 0.5 ml of an FMDV serotype O PanAsia strain at a titre of 10^5.9 TCID50/ml per animal. Two other cows (UQ59 and UQ61) were left in-contact with the inoculated cows (UQ58 and UQ60 respectively). All four cows were of Friesian-Holstein breed. Each cow was machine milked daily for the duration of the study (except on days 11 and 13) and fore-milk was also collected daily by hand from the quarters of each cow at morning milking. On each day the quarter milks were pooled and an aliquot of this 'whole' fore-milk, and of the whole machine milk, was separated by centrifugation into skim milk, cream and cellular components (cellular debris). In addition to milk samples, serum was collected at -3, 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 23 and 28 days post infection (dpi). Nasal swabs, mouth swabs (saliva) and "probang" samples were also collected from each cow on most days. Aliquots of these samples were also placed into buffer for RT-PCR. The clinical signs indicative of FMDV infection were monitored daily. Feet and teats were closely inspected, body temperatures were measured and milk yields recorded. One cow (UQ61) was euthanased on day 16.
due to lameness while the other three cows were killed on day 28. A wide range of tissue samples were collected at post-mortem for subsequent analysis.

Optimisation of the automated RNA extraction protocol for milk samples
The optimal RNA extraction protocol for milk components was determined in preliminary experiments using uninfected whole milk spiked with another FMDV PanAsia isolate. QIAGEN robotic apparatus was used to process the virus dilutions added to the extraction buffers: TRIzol Reagent® (Invitrogen), Lysis/Binding Buffer (Roche) and Buffer AL Lysis buffer (QIAGEN). An automated programme on a QIAamp® Virus BioRobot® 9604 extracted nucleic acid from each sample and a BioRobot® 3000 performed all subsequent liquid handling steps for the RT and PCR procedures by transferring volumes similar to those described previously (Reid et al., 2003). Other details of the reverse transcription procedure and PCR reaction mixture were as described previously (Reid et al., 2003). As a comparison between different robotic equipment, RT-PCR was also carried out on the virus dilutions added to TRIzol Reagent® and to Lysis/Binding Buffer using automated programmes on a MagNA Pure LC robot (Roche) which were similar to those described previously (Reid et al., 2003).

Quantitative real-time RT-PCR testing of milk, serum and nasal swab samples
RNA extraction and subsequent RT and PCR set up were performed on the fore- and machine whole milk from each cow and on the corresponding components of skim milk, cells and cream using the QIAGEN robotic apparatus. Quantification of FMDV was achieved using a dilution series of an RNA standard (RNA transcribed in vitro [MegaScript, Ambion] from a plasmid clone containing an internal ribosomal entry site [IRES] fragment of the FMDV genome run in parallel in each RT-PCR assay plate [Zhang et al., 2004]).

Detection of FMDV in tissues collected at post-mortem
Small pieces of several tissues including soft palate, pharynx, tonsil, and mammary gland sinus were collected at post-mortem (at 28 dpi) from animals UV58, UV59 and UV60 and quantitative RT-PCR was performed on these tissues using reagents from a MagNA Pure LC mRNA Extraction Kit II ([Tissues], Roche) with automated programmes on a MagNA Pure LC robot which were similar to those described previously (Zhang and Alexandersen, 2004).

Virus isolation
Comparative titrations of the samples were performed in primary calf thyroid cells (CTY; Snowden, 1966). The FMDV specificity of random samples producing a cytopathic effect (CPE) was confirmed by ELISA (Ferris and Dawson, 1988) and the titre of the virus stock was expressed as 50% tissue culture infective doses (TCID_{50}/ml; Kärber, 1979).

Dilution of FMDV in uninfected milk
Five samples of whole milk collected on days 3 and 8 of the experiment were diluted in log_{10} steps from undiluted to 10^{-7} in uninfected milk. Each dilution plus a negative milk control was inoculated onto CTY cell culture monolayers and tested by the optimised real-time RT-PCR procedure in order to compare the end-point detection limit of FMDV in milk of the two procedures.

Effect of temperature upon the ability of RT-PCR and VI to detect FMDV in milk
The temperature stability of FMDV in milk was tested by RT-PCR and VI on whole and skim milk spiked with an FMDV isolate. Aliquots were stored at 4°C, room temperature and at 37°C for analysis by quantitative RT-PCR and VI. A thermal cycler also heated aliquots of the whole and skim milk at 72°C for 5 sec, 15 sec, 25 sec, 1 min and 5 min and at 95°C for 5 sec. The ability of the assays to detect FMDV in milk samples that had been treated with temperature conditions that mimic pasteurisation was also investigated. Aliquots of infected whole milk samples from the experiment (pre-pasteurisation) were subjected to a pasteurisation programme in a thermal cycler of 4°C for 5 min followed by 72°C for 25 sec and stored at -80°C. The pre- and post-pasteurisation samples were tested by the VI and optimised quantitative real-time RT-PCR procedures.

Effect of a common preservative solution on the performance of real-time RT-PCR
An experiment investigated whether addition of a milk preservative solution (PS) commonly used in the UK (c8.3% Bronopol/ c25.0% Kathon CG, Wychem Limited, UK) to FMDV-infected milk inhibited detection of viral genome by the real-time RT-PCR. The PS was added (0.25, 0.5 and 1.0 % v/v, respectively) to three aliquots of ‘spiked’ whole milk (protocols recommend that 50 µl of PS is added per 10 ml of sample solution). No preservative was added to a fourth ‘spiked’ whole milk aliquot. The aliquots were separated by centrifugation into skim milk, cream and cell components and together with the whole milk component were tested by RT-PCR.
Results:

Experimental infection of dairy cows with FMDV

All four cows developed severe clinical signs consistent with FMD (Figure 1). Epithelial lesions first appeared on the feet and udder of the directly inoculated cows before the in-contact animals. Both inoculated animals had lesions on all feet by day 2 and lesions appeared on all teats of the donors by day 3 and 5 respectively. Lesions were observed on the feet of the in-contact cows by days 4 to 5 and 5 to 6 respectively (Figure 1). Body temperature was monitored daily. All cows showed evidence of short-lived pyrexia (temperature > 40°C) on 2, 1 and 5 dpi for cows UV58, UV60 and UV61 respectively. Animal UV59 had a maximum body temperature of 39.4°C on day 5 post infection.

There was generally a close correlation between the results of the RT-PCR and VI on the serum and nasal swabs. Virus was first detected in the serum of both inoculated cows by RT-PCR on day 1 (days 1 and 2 by VI) and in the serum of both in-contact cows using both procedures on day 4 (Figure 1). FMDV was detected daily in serum for a period of 3 to 5 days in cows UV58, UV59 and UV61. Cow UV60 similarly showed a daily positive response lasting 3 days but FMDV was subsequently detected in the serum of this cow on some days by RT-PCR and VI.

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Nasal swabs from all cows were positive by RT-PCR on all days from 2 to 10 inclusive and positive by VI in at least two cows on days 2 through to 7. Virus was not isolated in the nasal swabs collected after day 7 but low copy numbers were detected by RT-PCR in the nasal swab of cow UV58 on day 23 and in the swab from cow UV60 on day 18. Mouth swabs (saliva) of cows UV58, UV59 and UV60 were positive by VI on day 2 and virus was isolated in cell culture from the swabs of all four cows on day 4 (data not shown). “Probang” samples of all four cows were positive on days 2 and 4 and virus was isolated from the “probangs” of cow UV58 through to day 28 (data not shown).

**Figure 1:** Infection of 4 dairy cows (UV58-61) with FMDV. Body temperature and daily milk yield were monitored over the duration of the study (4 to 28 days post infection [dpi]). Animals were also observed daily for vesicular lesions indicative of FMDV infection: the timing (dpi) of the first occurrence of these lesions at three epithelial sites is indicated (oral lesions not shown for directly inoculated cattle). The presence of FMDV in serum and nasal swab samples was monitored by real time RT-PCR (●) and virus isolation (○). Values shown are log10 FMDV copy number and log10 FMD viral titre for RT-PCR and VI methods respectively.
Optimisation of automated real-time RT-PCR for detection of FMDV in milk
The performance of two robotic systems (QIAGEN QIAamp® Virus BioRobot® 9604: Figure 2A and
MagNA Pure LC: Figure 2B) to prepare RNA template from milk samples was compared using 3
proprietary extraction/lysis buffers (QIAGEN AL buffer was not tested on the MagNA Pure LC robot).
The best recovery of FMDV spik ed into milk samples (Figure 2) was obtained using TRIzol Reagent®
with the QIAGEN instrumentation. Interestingly, although the Roche Lysis/Binding buffer was able to
recover RNA using the MagNA Pure LC (Figure 2B), this buffer failed to produce any amplifiable FMDV
template on the QIAGEN robot (Figure 2A).

Figure 2: Optimisation of RNA template extraction methods for detection of FMDV in milk. The
ability of real-time RT-PCR to detect a titration series of FMDV O/UKG 12/2001 in milk was compared
using the QIAGEN robots (QIAamp® Virus BioRobot® 9604/3000: Figure A) or a MagNA Pure LC
(Roche: Figure B). Samples were presented to the robots in Lysis/Binding Buffer (Roche: ▲), TRIzol®
Reagent (Invitrogen: ●) or Buffer AL Lysis buffer (QIAGEN: ○). Values shown are the CT values
obtained by RT-PCR.

Detection of FMDV in milk by quantitative real-time RT-PCR and VI
Temporal changes in FMDV copy number determined by RT-PCR and the VI titre are shown in Figure
3 for the fore- and machine whole milk and skim milk fractions from the four cows. The RT-PCR and
VI results again generally correlated closely. The presence of FMDV in the milk coincided with the
onset of, but did not precede, the expression of clinical signs in the cows. The earliest day that FMDV
was consistently detected was day 2, day 3, day 5 and day 4 for UV58, UV60, UV59 and UV61
respectively. The peak amount of FMDV in the milk appears to correlate with FMDV loads detected in
serum. Interestingly, UV60 had detectable FMDV in milk by RT-PCR but not VI. FMDV was detectable
in milk for up to 23 dpi (in UV58) in contrast to the FMDV viraemia which only lasted 4-6 days. Figure
3 shows secondary peaks of FMDV detection particularly evident for RT-PCR in the whole milk and
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3 shows secondary peaks of FMDV detection particularly evident for RT-PCR in the whole milk and
skim preparation of fore- and machine milk from cows UV58, UV59 and UV61. No significant
secondary peaks of FMDV infectivity were obtained by VI. Virus was isolated in culture from the cell
fraction of fore-milk from cows UV58, UV59 and UV61 on most of the days from 2 to 9 and from cow
UV58 through to day 18 (data not shown). RT-PCR was positive on the cell fraction from all cows but
with a reduced copy number in this fraction from cow UV60 compared to the other cows. The results
from the machine milk cell fractions were similar to those of the fore-milk cell fraction although the
cell fraction from cow UV60 produced a CPE in cell culture (data not shown). Virus was detected by
both procedures in the cell fraction of the machine milk of cow UV58 up to day 18. Cream separated
from fore- and machine milk from cows UV58, UV59 and UV61 was also positive by VI and RT-PCR
but only cream separated from the machine whole milk of cow UV60 was positive. Virus was not
found by either method in the cream component of the fore-milk from this cow (data not shown).
In Figure 3, detection of FMDV in milk samples collected from 4 experimentally infected cows (UV58-61). FMDV was detected by real-time RT-PCR (●) and virus isolation (○) in whole and skim milk fractions of fore-milk and machine milk collected at regular intervals after experimental infection (dpi). Values shown are \( \log_{10} \) FMDV copy number and \( \log_{10} \) TCID\(_{50}\)/ml FMD viral titre for RT-PCR and VI methods respectively.

In order to determine whether FMDV partitions into the different milk components, the ratio of FMDV recovered in the skim, cream and cellular fractions to that detected in the whole milk was calculated (Figure 4). This showed that a high percentage of virus was recovered in the cream component compared with the other components.

Detection of FMDV in tissues collected at post-mortem
Virus was detected in the soft plate of cows UV58 and UV59, in the pharynx of cow UV59 and in tonsil (UV58), medial lymph node (UV59 and UV60), mammary lateral lymph node (UV59) and retro pharyngeal lymph node (UV58). No virus was detected in the mammary glands or in the other tissues (data not shown).

Dilution of FMDV in uninfected milk
In two of the dilution series, RT-PCR was approximately 100-fold more sensitive than VI for detection of FMDV. RT-PCR was approximately 10-fold more sensitive than VI in two dilution series and the two procedures had an equivalent sensitivity for detection of FMDV in the fifth dilution series (data not shown).

Effect of temperature on the ability of RT-PCR and VI to detect FMDV in milk
The effect of incubating whole milk samples spiked with FMDV at different temperatures is shown in Figure 5. Similar RT-PCR and VI data was obtained for skim milk (data not shown). The presence of FMDV detected by both RT-PCR and VI declined rapidly at 95°C and 72°C, although in contrast to VI, RT-PCR was still able to detect FMDV in milk after treatment at 72°C for 5 min. Furthermore, RT-PCR was able to detect FMDV in milk incubated at 37°C and room temperature for a longer period of time. FMDV can be detected by both assays after two week incubation at 4°C, although a significantly reduced amount of virus is recovered after this period.
The results for temperature stability were consistent with those obtained from an experiment which mimicked the effects of pasteurisation on milk samples obtained from infected cattle (data not shown). Heat treatment at 72°C for 15 sec, eliminated the ability of VI to detect FMDV but merely reduced the copy number obtained by RT-PCR compared with pre-pasteurisation determinations.

**Effect of preservative treatment on the performance of real-time RT-PCR**

Preservative treatment at concentrations ranging from 0.25% v/v to 1.0% v/v in infected milk did not have a significant inhibitory or deleterious effect on the ability of the RT-PCR to detect FMDV in any of the whole milk, skim, cell and cream fractions of that milk (data not shown).
Figure 5: Effect of temperature on the ability of real-time RT-PCR (Figure A) or virus isolation (Figure B) to detect FMDV in milk. Samples of milk were spiked with O/UKG 12/2001 and were incubated at 95 °C (●), 72 °C (▲), 37 °C (■), room temperature ~ 22 °C (♦) and 4 °C (♦).

Discussion:
The primary aim of this study was to assess the feasibility of using real-time RT-PCR to detect FMDV in milk. In order to select the most appropriate automated extraction protocol for use with milk samples, initial experiments were performed to compare the recovery of FMDV RNA template from milk using different robotic systems with 3 separate sample lysis buffers. Proprietary lysis buffers: Lysis/Binding Buffer and Buffer AL were able to recover RNA template successfully using their respective automated systems, although samples prepared in TRIzol Reagent® generated the highest signal with the QIAGEN robot. Therefore, the QIAGEN robotic apparatus was selected for the template extraction and subsequent liquid handling steps in this study. In addition to favourable FMDV RNA yields, this robot system benefits from a high sample throughput.

Suitable material for the evaluation of RT-PCR was generated by experimental infection of 4 dairy cows (2 by direct inoculation and 2 by contact) with FMDV type O UKG 34/2001. Fore- and machine milk were compared as both might be submitted for diagnostic evaluation, but there was little difference between the results obtained with these sample types. All four cows quickly developed multiple signs of clinical disease following inoculation. The clinical signs correlated with the high level of viraemia determined by real-time RT-PCR and VI and coincided with the presence of FMDV in the “probangs”, saliva and nasal swabs; results consistent with those achieved from previous
experimental infection of cattle with FMDV (Alexandersen et al., 2003). Milk samples (and milk fractions: skim, cream and cellular pellet) were tested by quantitative real-time RT-PCR assay, targeting the 5’untranslated region of FMDV (Reid et al., 2003). These milk samples were also tested in parallel by VI (Snowdon, 1966) in order to compare the performance of RT-PCR with an established diagnostic method with high sensitivity. Broad agreement was found between the ability of RT-PCR and VI to detect FMDV in samples of fore- and machine milk. The similarity between these two diagnostic approaches was particularly evident at early time-points after infection. At later time-points (typically >10 dpi), VI was less likely than RT-PCR to detect FMDV, probably due to the development of neutralising antibodies in these milk samples. A further benefit of RT-PCR over VI was the lower detection limit of the molecular assay on a dilution series of naturally infected milk. These data suggest that RT-PCR could potentially detect the presence of a single FMDV infected animal within an otherwise normal herd on the basis of testing an aliquot of milk from the combined bulk. RT-PCR was able to detect FMDV in milk collected from an individual cow that had been diluted 10,000-fold. To aid in the diagnosis of FMD further, RT-PCR could be used in parallel with assays to detect FMDV antibodies in milk (Armstrong and Mathew, 2001).

The excretion pattern of FMDV in milk as detected by VI paralleled results of previous studies (de Leeuw et al., 1978; Blackwell et al., 1982) and the presence of FMDV in the mammary gland up to 4 days after experimental infection has been documented by Blackwell and Yilma (1981). Interestingly, by RT-PCR the FMDV excretion profiles for one animal (UV58) showed a biphasic pattern: an initial period of FMDV excretion that lasted 6 days (2-7 dpi) followed by a subsequent peak (11-23 dpi). These data suggest that the mammary glands (or associated tissues) are a site of local replication of FMDV. However, there was no evidence for the persistence of FMDV in mammary tissue at 28 dpi despite the low copy numbers of RNA detected in the mammary lateral lymph node of cow UV59 during the post-mortem analysis.

In addition to whole skim milk, the RT-PCR was able to detect FMDV in cream and cellular fractions. In common with previous studies (Blackwell and Hyde, 1976), the RT-PCR data demonstrated that FMDV readily partitions into the cream fraction of milk. The ability of the real-time RT-PCR to detect FMDV in whole and skim milk after incubation at various temperatures was also investigated. As expected, and as reported in previous studies (Blackwell and Hyde, 1976; Tomasula and Konstance, 2004), VI was unable to detect viable virus after heat treatment at 95°C or 72°C. The results from this study show that although heat treatment reduces the amount of FMDV detected, RT-PCR was still able to detect FMDV genome in whole and skim milk that had been incubated at 95°C or under conditions that simulated pasteurisation. Therefore, RT-PCR may be an appropriate method for the detection of FMDV in dairy products made from pasteurised milk. In this study, no attempt was made to assess whether the pasteurised milk contained viable FMDV that could generate clinical disease in susceptible animals as shown previously (Blackwell and Hyde, 1976; Walker et al., 1984). These stability studies also showed that RT-PCR could successfully detect FMDV in milk after incubation at 37°C, room-temperature (approximately 22°C) and 4°C for extended periods. Addition of the preservative solution to FMD-infected whole milk also had no significant affect on the sensitivity of the RT-PCR when all components were tested, indicating that the ability of the RT-PCR to detect FMDV in bulk milk samples would not be impaired by this preservative.

**Conclusions:**
- Automated RT-PCR would be an excellent procedure for the laboratory detection of FMDV in milk.
- RT-PCR was more sensitive than VI and was able to detect FMDV in milk samples for longer periods after infection.
- The data from this limited number of animals did not provide any evidence that the presence of FMDV in milk could be used as a reliable preclinical indicator of FMD.

**Recommendations:**
- Automated RT-PCR could be used to test bulk tank milk samples and/or to screen herds for the presence of FMDV.
- Further experiments or field studies involving larger numbers of in-contact or naturally infected cattle are required in order to clearly define the relationship between the presence of FMDV in milk and the presentation of clinical signs.

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