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Detection of foot-and-mouth disease virus: comparative diagnostic sensitivity of two independent real-time reverse transcription-polymerase chain reaction assays

Donald P. King,¹ Nigel P. Ferris, Andrew E. Shaw, Scott M. Reid, Geoff H. Hutchings,
Angelica C. Giuffre, John M. Robida, Johnny D. Callahan,
William M. Nelson, Tammy R. Beckham

Abstract. Rapid and accurate diagnosis is central to the effective control of foot-and-mouth disease (FMD). It is now recognized that reverse-transcription polymerase chain reaction (RT-PCR) assays can play an important role in the routine detection of FMD virus (FMDV) in clinical samples. The aim of this study was to compare the ability of 2 independent real-time RT-PCR (rRT-PCR) assays targeting the 5' untranslated region (5'UTR) and RNA polymerase (3D) to detect FMDV in clinical samples. There was concordance between the results generated by the 2 assays for 88.1% (347 of 394) of RNA samples extracted from suspensions of epithelial tissue obtained from suspect FMD cases. The comparison between the 2 tests highlighted 19 FMDV isolates (13 for the 5'UTR and 6 for the 3D assay), which failed to produce a signal in 1 assay but gave a positive signal in the other. The sequence of the genomic targets of selected isolates highlighted nucleotide substitutions in the primer or probe regions, thereby providing an explanation for negative results generated in the rRT-PCR assays. These data illustrate the importance of the continuous monitoring of circulating FMDV field strains to ensure the design of the rRT-PCR assay remains fit for purpose and suggest that the use of multiple diagnostic targets could further enhance the sensitivity of molecular methods for the detection of FMDV.

Key words: Foot-and-mouth disease virus; real-time reverse transcription-polymerase chain reaction; 5' untranslated region.

Foot-and-mouth disease (FMD) is a contagious viral disease affecting cloven-hoofed livestock, principally cattle, pigs, sheep, and goats. The 2001 outbreak in

the United Kingdom highlighted the devastating impact of FMD in a country previously free from the disease. This outbreak lasted for approximately 7 months, requiring the slaughter of 6.5 million animals for its control, and is estimated to have cost £8 billion to the UK economy. Rapid and accurate diagnosis plays an important role for the implementation of effective measures to control the spread of disease. The causative agent, FMD virus (FMDV), is a highly variable RNA virus that exists as 7 immunologically distinct serotypes, which in most cases can be further subdivided into a number of topotypes.⁵ Recently, real-time reverse transcription-polymerase chain reaction

From the Institute for Animal Health, Pirbright Laboratory, Pirbright, Woking, Surrey, UK (King, Ferris, Shaw, Reid, Hutchings), Foreign Animal Disease Diagnostic Laboratory, National Veterinary Services Laboratories, USDA/APHIS, Plum Island Animal Disease Center, Greenport, NY (Giuffre, Robida, Beckham), and Tetracore Inc., Gaithersburg, MD (Callahan, Nelson). Current address Beckham: Department of Homeland Security, Plum Island Animal Disease Center, Greenport, NY.

¹Corresponding Author: Donald P. King, Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Woking, Surrey, GU24 0NF, UK. donald.king@bbsrc.ac.uk

(rRT-PCR) assays^{2,6-9} have been developed with the capability of detecting FMDV in a variety of sample matrices. These assays characteristically possess both high analytical sensitivity and high specificity. The ability to detect diverse FMDV strains (pan-reactivity) is an additional requirement for any routine FMD diagnostic assay. Although many of the published rRT-PCR assays are reported to be able to recognize members of all 7 FMDV serotypes, detailed evaluation of diagnostic sensitivity (D-SN) in direct comparison with other diagnostic methods such as virus isolation (VI) and antigen-enzyme-linked immunosorbent assay (Ag-ELISA) has currently been published for only 1 rRT-PCR assay.¹⁰ This previous study concluded that the rRT-PCR method used had superior sensitivity to established diagnostic methods, for FMDV was detected in 18% more samples compared with VI and Ag-ELISA combined.¹⁰

As part of the on-going validation for molecular methods, the aim of this current investigation was to compare in parallel the ability of 2 rRT-PCRs to detect a diverse selection of FMDV isolates. These assays, both in routine use by national reference laboratories, were designed to target 2 different conserved regions of the FMD viral genome: the internal ribosomal entry site located in the 5' untranslated region (5'UTR)⁸ and viral RNA polymerase (3D).² The laboratory work was performed during 2004 by staff from the Foreign Animal Disease Diagnostic Laboratory (FADDL, USDA/APHIS, Plum Island Animal Disease Center, Greenport, NY) and the Institute for Animal Health (IAH, Pirbright, Woking, Surrey, UK) using samples of epithelial tissue ($n = 394$) submitted to the FAO World Reference Laboratory for FMD (IAH, Pirbright).

The epithelial tissues were collected from suspect FMDV cases during a 40-year period (1964–2004) from 69 countries. Vesicular epithelial tissue is the preferred sample for diagnosis from infected animals because it is rich in virus during the acute stage of disease. Samples were selected from available material to ensure adequate coverage of the inter- and intratypic genetic variation of FMDV. In 81.2% (320 of 394) of cases, epithelial suspensions (ES) were prepared³ at the time of sample receipt and were stored in the interim at -80°C , whereas the remainder were processed at a later date. The suspensions had been previously assayed for FMDV by using VI and Ag-ELISA.³ The Ag-ELISA was also used to confirm the presence of FMDV in VI samples showing cytopathic effects.³ The archived samples used in the study covered all 7 FMDV serotypes: 181 type O, 56 type A, 31 type Asia 1, 13 type C, 19 type SAT 1, 24 type SAT 2, and 7 type SAT 3. Suspect samples where no FMDV had been detected by VI or Ag-ELISA (classified as “no virus detected” [NVD; $n = 63$]) were also examined.

Original ES stored at -80°C were used as the starting material for RNA extraction. Template RNA was prepared^a from 140 μl of the ES and eluted in 40 μl . A total of 2.5 μl of RNA was tested by a commercial 1-step rRT-PCR assay targeting 3D^{b,c} as previously described.² The RNA samples were frozen at -80°C until tested with a second rRT-PCR targeting the 5'UTR.⁸ Briefly, cDNA was synthesised^d with 6 μl of RNA in a total reaction volume of 15 μl as previously described.⁹ Polymerase chain reactions were prepared by using a robot^e with 7 μl of cDNA, TaqMan mastermix^f and primers and probe. Amplification conditions^g used were as previously described.⁹ Preliminary experiments indicated that a single freeze-thaw of the RNA template did not consistently affect the cycle threshold (C_T) values obtained by more than 1 cycle in the 5'UTR rRT-PCR (data not shown). A positive control consisting of pooled RNA extracted from the FMDV reference strain O₁ Manisa (TUR 8/69) was included in every assay run.

A comparison of the rRT-PCR results for 394 ES are shown in Fig. 1 separated into the individual FMDV serotypes (and NVD) and summarized for all the samples tested in Fig. 2. The C_T used to designate positive samples was different between the 2 assays: <40 for the 5'UTR assay¹⁰ and <45 for the rRT-PCR-targeting FMDV 3D assay.² Cycle threshold values falling between those of the diagnostic cut-off and absolute negative of the rRT-PCR (no C_T) were designated as borderline (shaded gray area on Figs. 1, 2).

There was agreement in the diagnostic result (either positive or NVD) produced between the 2 rRT-PCR assays for 88.1% (347 of 394) of the ES. These 347 samples consisted of 322 ES that were positive in both rRT-PCRs and 25 ES where both assays failed to detect FMDV. Of concern, the comparison between the 2 tests also highlighted 19 FMDV isolates (13 for the 5'UTR and 6 for the 3D assay; Fig. 2), which failed to produce a signal in one assay but gave a positive signal in the other assay. The region of the FMDV genome targeted was sequenced for 12 selected isolates to elucidate the reason why these viruses failed to generate a signal in the rRT-PCR. Fragments encompassing the diagnostic target were amplified by RT-PCR with primers 5'-CGT CHG CGC ACG AAA CGC-3' and 5'-RCG ATR AAR CAG TCR GTY R-3' for the 5'UTR or 5'-GAC AAA GGT TTT GTT CTT GGT CA-3' and 5'-TCA CCG CAC ACG GCG TTC A for the 3D region.⁷ Polymerase chain reaction products were directly sequenced on both strands.^h

Sequence analysis revealed virus isolates with nucleotide substitutions that could explain a failure of the rRT-PCR. Ten serotype O viruses were missed by the 5'UTR assay. These consisted of closely related isolates collected from Wales during the 2001 epidemic

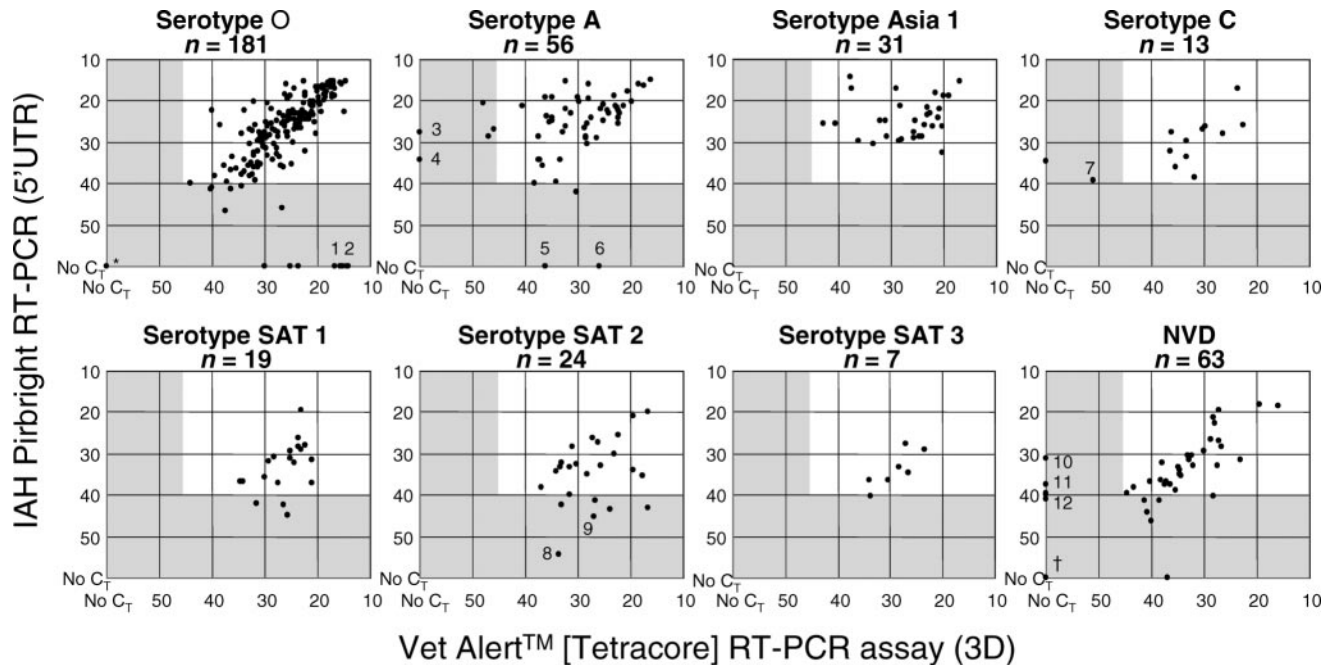


Figure 1. Comparison between cycle threshold (C_T) values obtained by real-time reverse-transcription polymerase chain reactions (rRT-PCRs) targeting the 5' untranslated region and 3D regions. Results are shown for 394 epithelial samples and are separated into the 7 foot-and-mouth disease virus (FMDV) serotypes determined by antigen–enzyme-linked immunosorbent assay (Ag-ELISA) of original material or cell culture grown antigen. Samples that failed to generate a cytopathic effect in culture or a signal in Ag-ELISA were designated as “no virus detected.” Positive rRT-PCR values are plotted in the white area, whereas borderline results that fell between the cut-off C_T value defined for the respective rRT-PCR assays and absolute negative (no C_T) are shown in the gray area. Sequences were obtained for selected isolates where there was poor concordance between the 2 assays: 1) UKG 13708/2001, 2) UKG 14221/2001, 3) SYR 9/2002, 4) BHU 41/2002, 5) GHA 4/96, 6) BKF 2/94, 7) PHI 1/94, 8) LBR 1/74, 9) SAU 2/2000, 10) SYR 7/2002, 11) SRL 13/88, and 12) BHU 32/04. * represents results for 2 samples: GRE 1/96, where FMDV/O was detected by Ag-ELISA but not by virus isolation (VI) or either rRT-PCR method; and IRQ 5/94, which was originally VI positive (FMDV/O), but insufficient archived sample was available to attempt reisolation at the time of this study. † represents identical negative results for 23 samples that generated “no C_T ” results with both rRT-PCR assays.

in the United Kingdom. Identical sequences were obtained for 2 of these isolates. As reported elsewhere,⁴ the failure of the assay can be explained by 3 nucleotide substitutions in the TaqMan probe region. The 3 remaining samples (A GHA 4/96, A BKF 2/94, and NVD GHA 2/2002) completely missed by the 5'UTR assay were of west African origin. It was possible to obtain sequences for 2 of these isolates which were identical and highlighted 3 and 1 nucleotide substitutions with the forward primers and reverse primers, respectively. Figure 3 also shows the sequences of 2 SAT 2 isolates (which generated borderline C_T values in the 5'UTR assay), which have further nucleotide mismatches with the probe and primers. For the 3D RT-PCR target, 6 sequences were obtained from A, C, and NVD samples. All these sequences revealed nucleotide mismatches that might explain the poor performance of these samples in the 3D rRT-PCR assay. Retrospective rRT-PCR analysis of cell culture supernatant derived from in vitro passage of 1 of these isolates (A BHU 41/2002) showed that this material (presumably containing a higher amount of virus than the

ES) could be detected by the assay, although the C_T value generated was higher (33.36) than the corresponding value (17.25) of the 5'UTR assay (NB: higher C_T values denote lower signal in the rRT-PCR).

Analysis of the mean differences in C_T between the 2 assays for samples was performed to assess whether there was any serotype bias in either of the rRT-PCRs. When all the samples were analyzed together, this analysis (Table 1) showed that the overall difference between the 2 assays was not significant. However, there were significant differences in mean C_T values between the 2 assays at the serotype level. In particular, the 5'UTR assay performed less well against FMD viruses of the SAT serotypes (as previously reported¹), whereas serotype A viruses produced proportionally less signal in the 3D rRT-PCR. These differences can also be seen in the scatter plots in Fig. 1. It is possible to speculate that the relatively poor signal reflects the presence of further nucleotide changes in the primer or probe sites (in addition to those described above), which leads to a reduced efficiency of the assay but not to a complete loss of signal. As might be

VetAlert™ Tetracore Assay (3D)

		Positive		NVD
		$C_T > 45 - < 55$		
IAH Assay (5'UTR)	Positive	322	3	6
	$C_T > 40 - < 55$	23	0	2
	NVD	13	0	25

Figure 2. Summary of the results generated by the 2 real-time reverse transcription-polymerase chain reaction (rRT-PCR) assays for epithelium samples ($n = 394$). Borderline results that fell between the cut-off cycle threshold (C_T) value defined for the respective rRT-PCR assays and absolute negative (no C_T) are tallied in the middle gray boxes. For instance, 23 samples that were positive by using the 3D assay gave a borderline reading with the 5' untranslated region (5'UTR) rRT-PCR. NB: the 5'UTR rRT-PCR uses a more conservative cut-off ($C_T < 40$) than that used for the 3D rRT-PCR assay ($C_T < 45$).

expected, some of the viruses that were poorly detected in either of the assays were closely related to each other, suggesting that common sequence mismatches were present. However, the serotype A viruses that performed poorly in the 3D rRT-PCR were from at least 4 unrelated phylogenetic groups (determined by analysis of VP1 sequences held at IAH).

In this study, although VI and Ag-ELISA assays for FMDV were not performed at the same time as the

Table 1. Comparison among C_T values obtained by 3D- and 5'UTR-specific rRT-PCRs. Student's paired t -test was used to assess whether the differences between the assays was significant.†

Serotype	n ‡	Mean difference in C_T between assays§	Student's t -test P value
O	169	0.01	0.98
A	52	4.48*	3.30×10^{-6}
Asia 1	31	2.64	0.07
C	12	2.45	0.13
SAT 1	19	-6.86*	4.44×10^{-5}
SAT 2	24	-7.45*	1.94×10^{-4}
SAT 3	7	-4.95*	2.12×10^{-3}
All samples	314	0.16	0.67

* Difference between the assays significant at $P < 0.05$.

† C_T = cycle threshold; 3D = 3 dimensional; 5'UTR = 5' untranslated region; rRT-PCR = real-time reverse-transcriptase PCR.

‡ Samples that gave a negative result (no C_T) in either or both rRT-PCR assays were excluded from this analysis.

§ Calculated by subtracting the C_T value of the 5'UTR assay from the 3D assay. Cycle threshold values are inversely proportional to signal in the rRT-PCR; therefore, positive and negative numbers in this column represent increased signal for the 5'UTR or 3D rRT-PCR assays, respectively.

rRT-PCR, it is possible to get some indication as to the performance of the rRT-PCR in comparison with these established assays, which many researches consider to be the "gold standard." Using data from samples where the ES were prepared at the time of sample receipt ($n = 320$), the sensitivity values were 95.4% for the 5'UTR and 97.7% for the 3D rRT-PCR. Positive rRT-PCR results were also obtained for a number of the samples that were originally classified as negative by VI and Ag-ELISA (as shown in the NVD plot in Fig. 1). These results, which show that rRT-PCRs

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[1] UKG 13708/2001 CACTTTAAGGTGACACTGATACTGGTACTCAACCCTGGTGACAGGCTAAGGATGCCCTTAAAGTACCCGAGGTAACACGCGACACTCGGGATCTG
[2] UKG 14221/2001 CACTTTAAGGTGACACTGATACTGGTACTCAACCCTGGTGACAGGCTAAGGATGCCCTTAAAGTACCCGAGGTAACACGCGACACTCGGGATCTG
[5] GHA 4/96 CACCTTCAGGTAACACTGACTGGTACTTGGTTTCTGGAGACAGGCTAAGGATGCCCTTCAGGTACCCGAGGCAACAAGAGACACTTGGGATCTG
[6] BKF 2/94 CACCTTCAGGTAACACTGACTGGTACTTGGTTTCTGGAGACAGGCTAAGGATGCCCTTCAGGTACCCGAGGCAACAAGAGACACTTGGGATCTG
[8] LBR 1/74 CACTTTAAGGTGACACTGGACTGGTACTTGGTTTCTGGAGACAGGCTAAGGATGCCCTTCAGGTACCCGAGGTAACACGCGACACTCGAGATCTG
[9] SAU 4/2000* CACTTTAAGGTGACACTGACTGGTACTTGGTTTCTGGAGACAGGCTAAGGATGCCCTTCAGGTACCCGAGGTAACACGCGACACTCGAGGATCTG
    
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[3] SYR 9/2002 ACTGGGTTTTACAAACCTGTGATGGCTCGAAGACCCCTCGAGGCCATCCTCTCCTTTGCACGCCGTGGGACCATA CAGGAGAAGTTGATCTGTGGCGGACTCGC
[4] BHU 41/02 ACTGGGTTTTACAAACCTGTGATGGCTCGAAGACCCCTCGAGGCCATCCTCTCCTTTGCACGCCGTGGGACCATA CAGGAGAAGTTGATCTGTGGCGGACTCGC
[7] PHI 1/94† ACAGGTTTTACAAACCTGTGATGGCTCGAAGACCCCTCGAGGCCATCCTCTCCTTTGCACGCCGTGGGACCATA CAGGAGAAGTTGATCTCCGTGGCAGGACTCGC
[10] SYR 7/2002 ACTGGGTTTTACAAACCTGTGATGGCTCGAAGACCCCTCGAGGCCATCCTCTCCTTTGCACGCCGTGGGACCATA CAGGAGAAGTTGATCTGTGGCGGACTCGC
[11] SRL 13/88 ACTGGGTTTTACAAACCTGTGATGGCTCGAAGACCCCTCGAGGCCATCCTCTCCTTTGCACGCCGTGGGACCATA CAGGAGAAGTTGATCTCCGTGGCAGGCTCGC
[12] BHU 32/2004 ACTGGGTTTTACAAACCTGTGATGGCTCGAAGACCCCTCGAGGCCATCCTCTCCTTTGCACGCCGTGGGACCATA CAGGAGAAGTTGATCTCCGTGGCAGGCTCGC
    
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Figure 3. Sequences for 12 virus isolates where there was poor concordance between the real-time reverse transcription-polymerase chain reaction (rRT-PCR) assays targeting the foot-and-mouth disease virus (A) 5' untranslated region and (B) (3D) regions. The locations of the respective primer and probe recognition sites are underlined. Nucleotide substitutions that are not tolerated by the primer or probe sets are indicated (A G T C). * SAU 4/2000 was isolated from the same premises as SAU 2/2000. † PHI 1/94 is in the same phylogenetic lineage as the serotype C isolate (PHI 1/88) that failed to generate a signal in the 3D rRT-PCR.

can detect FMDV in samples that are negative by VI and Ag-ELISA, are consistent with findings from a previous study.¹⁰ They probably reflect that molecular methods have high analytical sensitivity and will detect FMDV even if the samples are of poor quality and do not contain live virus. A consequence of the superior performance of the rRT-PCR assays is that very low specificity values are generated for both assays (33.3% for the 5'UTR and 38.6% for the 3D rRT-PCR assays), suggesting that caution should be placed on the use of VI and Ag-ELISA as a gold standard for FMDV diagnosis. In contrast to the NVD samples, only 4 of the 334 samples that were originally reported as positive by a combination of VI and Ag-ELISA failed to produce a signal in either of the rRT-PCR assays. Two of these samples were recategorized for this analysis as NVD because repeat attempts to isolate virus from the original ES failed, suggesting that the samples either had degraded or had been incorrectly labeled. The third sample (serotype O GRE 1/96) was positive by Ag-ELISA but not by VI or by either rRT-PCR method. The fourth sample (serotype O IRQ 5/94) was originally VI positive, but insufficient archived sample was available to attempt reisolation.

In summary, the overall performance of the 2 rRT-PCR assays was similar. This current investigation using approximately 400 samples consisting of clinical material from suspect cases of FMD expands on a study previously carried out at the Australian reference laboratory for FMD,¹ where both 5'UTR and 3D assays were tested on a small selection of inactivated viruses. These results emphasize the importance of creating depth and diversity in assay systems used to detect economically important diseases such as FMD. This study suggests that using both of these rRT-PCR assays (recognizing independent regions of the FMD viral genome) in concert would increase D-SN in a suspect FMD sample. This would decrease the likelihood of a false-negative result secondary to sequence variability and add additional validity to the final diagnosis. Parallel and multiplex rRT-PCRs exploiting these improved assay characteristics are currently under development. However, even when used independently, these rRT-PCR assays offer superior sensitivity over established diagnostic tests. This report provides further confidence in the use of this method for routine diagnosis of FMD and re-emphasizes the effectiveness of rRT-PCR as a diagnostic tool for routine FMD detection and control.

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Sources and manufacturers

- a. Rneasy Mini-Kit, Qiagen, Valencia, CA.
- b. Vet-Alert, Foot-and-Mouth Disease, Tetracore Inc., Gaithersburg, MD.
- c. SmartCycler II (Cepheid), Sunnyvale, CA.
- d. MultiScribe reverse transcriptase, Applied Biosystems, Warrington, UK.
- e. BIOROBOT 3000 (Qiagen), Crawley, UK.
- f. 2xTaqMan mastermix, Applied Biosystems, Warrington, UK.
- g. MX4000, Stratagene, La Jolla, CA.
- h. Beckman CEQ 8000 DNA analysis system, Fullerton, CA.

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