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Diagnostic evaluation of a multiplexed RT-PCR microsphere array assay for the detection of foot-and-mouth disease virus and look-alike disease viruses

B. J. Hindson, S. M. Reid, B. R. Baker, K. Ebert, N. P. Ferris, L. F. Bentley Tammero, R. J. Lenhoff, P. Naraghi-Arani, E. A. Vitalis, T. R. Slezak, P. J. Hullinger, D. P. King

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Diagnostic evaluation of a multiplexed RT-PCR microsphere array assay for the detection of foot-and-mouth and look-alike disease viruses

Running title: Multiplexed RT-PCR detection of FMDV and look-alikes

Benjamin J. Hindson[1]*, Scott M. Reid[2], Brian R. Baker[1], Katja Ebert[2], Nigel P. Ferris[2], Lance F. Bentley Tammero[1], Raymond J. Lenhoff[1], Pejman Naraghi-Arani[1], Elizabeth A. Vitalis[1], Thomas R. Slezak[1], Pamela J. Hullinger[1] and Donald P. King[2]

[1]Lawrence Livermore National Laboratory, Livermore, California

[2]Institute for Animal Health, Pirbright Laboratory, Pirbright, Woking, Surrey, GU24 0NF, United Kingdom

*Corresponding author. Mailing address: Lawrence Livermore National Laboratory, P.O. Box 808, L-211, Livermore, CA 94551. Phone: (925) 423-8667. Fax: (925) 424-2778. E-mail: hindson1@llnl.gov

ABSTRACT

A high-throughput multiplexed assay was developed for the differential laboratory diagnosis of foot-and-mouth disease virus (FMDV) from viruses which cause clinically similar diseases of livestock. This assay simultaneously screens for five RNA and two DNA viruses using multiplexed reverse transcription PCR (mRT-PCR) amplification coupled with a microsphere hybridization array and flow-cytometric detection. Two of the seventeen primer-probe sets included in this multiplex assay were adopted from previously characterized real-time RT-PCR (rRT-PCR) assays for FMDV. The diagnostic accuracy of the mRT-PCR was evaluated using 287 field samples, including 248 (true positive n= 213, true negative n=34) from suspect cases of foot-and-mouth disease collected from 65 countries between 1965 and 2006 and 39 true negative samples collected from healthy animals. The mRT-PCR assay results were compared with two singleplex rRT-PCR assays, using virus isolation with antigen-ELISA as the reference method. The diagnostic sensitivity of the mRT-PCR assay for FMDV was 93.9% [95% C.I. 89.8-96.4%], compared to 98.1% [95% C.I. 95.3-99.3%] for the two singleplex rRT-PCR assays used in combination. In addition, the assay could reliably differentiate between FMDV and other vesicular viruses such as swine vesicular disease virus and vesicular exanthema of swine virus. Interestingly, the mRT-PCR detected parapoxvirus (n=2) and bovine viral diarrhea virus (n=2) in clinical samples, demonstrating the screening potential of this mRT-PCR assay to identify viruses in FMDV-negative material not previously recognized using focused single-target rRT-PCR assays.

INTRODUCTION

Foot-and-mouth disease (FMD) is a highly infectious and contagious vesicular disease affecting domestic and wild ruminants and swine caused by a single-stranded positive-sense RNA virus having seven distinct serotypes (A, Asia 1, C, O, SAT 1, SAT 2 and SAT 3) (17). FMD is endemic in many countries throughout the world, with serotype O having the highest prevalence, followed by serotype A (9). Early detection of the virus is critical to minimizing disease spread and the significant economic implications (12) resulting from the introduction of FMD into a country previously free of the disease. Diagnosis of FMD can be confounded by diseases with similar clinical signs ("look-alike" diseases), and by species where presentation of the disease is mild or indistinct (16). For the laboratory identification of FMDV, the *Office International des Epizooties* (OIE) recommends virus isolation (VI), antigen-ELISA (Ag-ELISA), and RT-PCR with detection by agarose gel electrophoresis or in real-time using TaqMan® fluorogenic probes (15).

Real-time PCR is widely used by diagnostic laboratories, to complement or as a replacement for more traditional detection methods. Two independent real-time reverse transcription polymerase chain reaction (rRT-PCR) assays for FMD laboratory diagnosis target the ribosomal entry site of the 5´untranslated region (5´UTR) (30) and the viral RNA polymerase gene (3D) (4) on the highly variable FMDV genome. The 5´UTR and 3D rRT-PCR assays were initially compared prior to their implementation in Australia (3). A subsequent in-depth comparative evaluation (14) was conducted to further evaluate the effectiveness of these assays; demonstrating a higher diagnostic sensitivity of the rRT-PCR assays over VI and/or antigen-ELISA (35), particularly when both assays were used in combination. Both assays are used routinely in combination at the Food and Agriculture Organization of the United Nations, World Reference Laboratory (FAO WRL) for FMD. rRT-PCR assays have also been reported for the detection of other viruses which cause vesicular disease of livestock including swine vesicular disease (SVD) (29), vesicular stomatitis (VS) (11, 28) and vesicular exanthema of swine (VES) (31) or symptomatic look-alike diseases including bluetongue (13, 27, 33), bovine viral diarrhea

(1, 2, 20, 22, 39), malignant catarrhal fever (37) and parapoxvirus (26).

Fluorescent probes for rRT-PCR detection have broad emission spectra which limit multiplexing capacity to the four or five discrete optical channels typically present in most commercial real-time PCR instruments. Therefore, simultaneous testing for FMDV and look-alike disease viruses by rRT-PCR would require many assays to be run in parallel, thereby increasing the demand on instrumentation and reagents which escalates costs. A single multiplexed screening test that simultaneously detects and differentiates FMDV from look-alike disease viruses would be desirable. Such a test could facilitate rapid and cost-effective screening of suspect FMD field samples for laboratory differential diagnosis, targeted FMD surveillance, or embedded foreign animal disease surveillance whilst conducting routine testing for endemic diseases.

Luminex xMAP technology is a multiplexed high-throughput detection system (38) with many applications for nucleic acid detection (6). The Luminex array offers up to 100 independent channels using microspheres (5.6 μm) embedded with varying ratios of two fluorescent dyes. User-defined surface modifications can include oligonucleotides, antibodies, peptides or other macromolecules. Typically, a mixed suspension of functionalized microspheres is mixed with the sample to bind analytes which are then labeled with a fluorescent reporter and analyzed using a specialized flow-cytometer. The assay provides end-point detection with qualitative results by comparing fluorescence responses (median fluorescence intensity, MFI) of each microsphere class to cut-off values. Recent nucleic acid applications of the Luminex array include the detection and differentiation of Classical swine fever virus from other pestiviruses (5), human respiratory viruses (18, 19, 21, 23), human papillomavirus (10, 32) and human influenza A virus typing (40).

This report describes a novel multiplexed RT-PCR microsphere array assay for the differential detection of FMDV from look-alike disease viruses. The development, optimization and analytical evaluation of this multiplex assay will be reported separately. The primary purpose of this study was to evaluate the diagnostic performance of the mRT-PCR assay for the detection of FMDV using a panel of

suspect field samples. The diagnostic sensitivities of the 3D and 5´UTR FMDV assays in mRT-PCR format were compared to rRT-PCR using VI with Ag-ELISA as the reference method. The detection of look-alike diseases in suspect FMD field samples is also reported.

MATERIALS AND METHODS

Samples. The panel comprised epithelia (true positive n= 213, true negative n=34) from suspect field cases of FMD submitted from 65 countries to the FAO WRL for FMD between 1965 and 2006 and included representatives of all seven serotypes of FMDV. In addition, 39 samples of true negative tongue epithelial were collected from healthy cattle at a UK abattoir. All sample testing and reference measurements were conducted at the FAO WRL for FMD. Epithelia were ground and suspended to generate a ~10% w/v suspension in phosphate buffer (0.04M, pH 7.6). The epithelial suspensions (ES) were centrifuged, the supernatant collected, then stored at -80ºC. FMDV serotype was determined by conducting an antigen-ELISA (8) on the original ES or after viral propagation in cell culture. Samples of look-alike viruses from the FAO WRL for FMD collection included SVDV, VESV, San Miguel sea lion virus (SMSV), caliciviruses isolated from a variety of species (including cetacean, bovine, feline, reptile, skunk) and vesicular stomatitis virus (VSV).

Nucleic acid extraction. Total nucleic acid was extracted from each ES by an automated procedure using a MagNA Pure LC (Roche, UK) as previously described (14, 35). Extracted samples (40 µL) were aliquoted (3×13 µL), stored at -80ºC and thawed once just before use.

rRT-PCR. Previously reported protocols for the individual 3D (4) and 5´UTR (34) rRT-PCR assays were modified for use in this study. Briefly, 25 µl reaction mixes (SuperScript™ III Platinum® One-Step qRT-PCR System (Invitrogen)) containing 20 pmol of each primer, 7.5 pmol of dual-labelled TaqMan® probe and 5 µl total nucleic acid were prepared in an optical reaction plate (Stratagene, Amsterdam, The Netherlands). For both targets, RT-PCR amplification was performed in an Mx4000 Multiplex Quantitative PCR System (Stratagene) as described previously (34).

mRT-PCR assay design. A schematic depiction of the mRT-PCR assay is shown in Figure 1. The RT-PCR uses eighteen biotinylated forward and unmodified reverse primer sets (17 for detection, 1 serves as a control). The sequences that comprise the multiplex assay are shown Table 1. The multiplex assay was designed to detect and differentiate FMDV from SVDV, VESV, Bovine Viral Diarrhea Virus (BVDV), Bluetongue Virus (BTV), Parapoxviruses (Orf virus, pseudocowpox virus, and bovine papular stomatitis virus), and Bovine Herpes Virus-1 (BHV-1). Primer-probe sequences for FMDV (4, 30) and BVDV (7, 22) were based on the work of others and adapted to the current multiplex format. All other sequences were designed at Lawrence Livermore National Laboratory (LLNL) using an approach that has previously been described (36). Additional computational analyses were performed to ensure specificity and reliability against all available data, including a BLAST-based comparison of each primer-probe set as a triplet against all sequences in GenBank to identify the targets that are predicted to produce a PCR or TaqMan reaction at 57 ºC for primer annealing and 67 ºC for probe annealing, where temperatures are derived from Primer 3 oligo T_M calculations. Optimal candidate primer-probe sets were forwarded to the bench screening phase for further down-selection. Amplicon sizes ranged from 95 -349 bp.

mRT-PCR primers and probes. All oligonucleotides used for mRT-PCR were synthesized by Integrated DNA Technologies (IDT, Coralville, IA) and HPLC-purified. Forward primers were functionalized with 5´ and internal biotin moieties. Reverse primers were unmodified. Probes were 5´ amino C6-modified with an 18-atom hexaethyleneglycol spacer. Lyophilized probe was dissolved in 2-(*N*-morphilino)ethanesulfonic acid (MES) to yield a stock concentration of 1 mM. Lyophilized forward and reverse primers were dissolved in TE buffer to yield a stock concentration of 1 mM. Working dilutions were prepared from the stock solutions as required.

Coupling of probe oligonucleotides to microspheres. xMAP® Multi-Analyte COOH Microspheres (Luminex Corp., Austin, TX) were covalently coupled to probe oligonucleotides using carbodiimide activation based on the manufacturer's protocol. Briefly, stock microspheres (1 mL, $1.25x10^7$

microspheres) were vortexed for 30 s, sonicated for 60 s, centrifuged at 8000 ×g for 5 min and the supernatant removed. The microspheres were then resuspended in MES (50 µL, 0.1M, pH 4.5), vortexed and sonicated. Probe (10 µL, 50 mM in MES) was added and the mixture vortexed. An aqueous solution of *N*-(3-dimethylaminopropyl)-*N´*-ethylcarbodiimide hydrochloride (EDC; 5 µL, 10 mg/mL) was added, vortexed, then gently agitated for 30 min in the dark. A second aliquot of EDC (5 µL, 10 mg/mL) was added, vortexed, then gently agitated for 30 min in the dark. Tween 20 (1 mL, 0.02% v/v) was added, vortexed, centrifuged, the supernatant removed, then repeated using SDS (1 mL, 0.1% m/v) then TE buffer (100 µL 1 mM Tris, 10 mM EDTA, pH 7.4). The probe-conjugated microspheres were resuspended in TE buffer (250 µL), vortexed, then stored at 4°C in the dark.

Microsphere mixture. A 21-plex microsphere suspension was prepared by combining individual stock suspensions (9 µL/class) with Tris-NaCl buffer (3 mL). The mixture was vortexed then enumerated using the Bio-Plex, targeting approximately 150 microspheres counts per class in 40 s. If required, additional microspheres from the individual stocks were added to ensure the concentrations of all classes were approximately equal.

mRT-PCR amplification. Each field sample was analyzed in duplicate by the multiplex assay. Amplification was performed using a one-step RT-PCR kit (SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase, Invitrogen). The reaction volume of 25 µL was comprised of nuclease-free water (0.95 µL), primer mix (3.6 µL), SuperScript™ III 2X reaction mix (12.5 µL), $MgSO_4$ (0.95 µL, 50 mM, Invitrogen), SuperScript™ III RT / Platinum® Taq Mix (1 µL), internal control armored RNA (1uL, ~100 copies) and template (5 µL). The internal control armored RNA was lysed by heating to 70°C for 4 min prior to addition to the mix. The final concentration of each primer and $MgSO_4$ was 0.4 µM and 3.5 mM, respectively. The RT-PCR thermal cycling protocol was 55°C for 30 min, 95°C for 2 min, then 35 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 15s, followed by 72°C for 2 min with a final 4°C hold.

Microsphere array hybridization. A wash-assay format was adopted to reduce variability of the response caused by non-specific hybridization of PCR products and fluorescent label. In a 96-well plate, RT-PCR product (1 μL) was added to a mixed suspension of probe-conjugated microspheres (22 μL) then placed in a thermal cycler and subjected to 95ºC for 2 min, 55ºC for 5 min, followed by a 4ºC hold. Tris-NaCl buffer (100 μL, 0.1M Tris, 0.2 M NaCl, 0.05% v/v Triton X-100, pH 8.0, Teknova) was added and the suspension was transferred to a 96-well vacuum filter plate (MABVN 1250 Multiscreen Filter Plate, Millipore). The suspension was vacuum-aspirated, and washed twice with Tris-NaCl buffer (2 ×100 μL). Stock streptavidin phycoerythrin (SAPE, 1 mg/mL, Caltag Laboratories) was diluted with Tris-NaCl to a working concentration of 3 μg/mL. SAPE (60 μL, 3 μg/mL) was added then incubated in the dark for 5 min. The suspension was vacuum-aspirated, washed once with Tris-NaCl (100 μL), re-suspended in Tris-NaCl (100 μL) then transferred to a 96-well round bottom plate for fluorescent detection. Nucleic acid extraction, PCR reaction assembly and PCR amplification were conducted in separate rooms. Likewise, hybridization and Bio-Plex detection were performed together in a separate room to minimize the likelihood of PCR contamination by amplicons.

mRT-PCR detection. Fluorescence detection of the processed microsphere suspension array was achieved using a Bio-Plex Workstation (Bio-Rad, CA) set to count a minimum of 100 events per microsphere class in a 50 μL Bio-Plex sample volume. The Bio-Plex Workstation is a specialized dual-laser flow cytometer integrated with XY microplate platform configured to analyze Luminex xMAP® microspheres in a 96-well plate format. The reporter PMT voltage was calibrated on the low setting with background subtraction enabled. The Bio-Plex workstation was validated and calibrated per the manufacturer's instructions. The resolution of the MFI was 0.5 units for all channels.

mRT-PCR assay controls. The multiplex assay incorporates four control channels integral to each reaction that are used to verify assay integrity. The negative control (NC) is a microsphere conjugated to *Thermotoga maratima*-derived oligonucleotide sequence (MT-7) that serves as a non-specific binding control in the multiplex PCR assay, and its response should remain consistently low (MFI≤80). The

fluorescence control (FC), biotinylated MT-7, confirms that fluorescent labeling with SAPE occurred and should exhibit a high response (MFI >1000). The instrument control (IC) comprises a Cy3-labeled MT-7 conjugate. As Cy3 and SAPE have similar fluorescence excitation and emission wavelengths, the IC confirms proper function of the reporter optics within the Bio-Plex flow cytometer (MFI>500). Armored RNA served as an end-to-end amplification control (AC) to reduce the probability of false negative and is utilized at low concentrations (100 copies/reaction) to generate a low-level response (MFI ≥20) which minimizes its competition with detection channels. The armored RNA (XenoRNA-01, Ambion, Austin, TX) is a proprietary 1070 nucleotide RNA transcript consisting of unique nucleotide sequences that possess no significant homology to the current annotated sequences in commonly used sequence databases including NCBI, Affymetrix, and Rosetta. Primers and probe-labeled microsphere for the AC are included in the multiplex primer mix and microsphere suspension, respectively.

mRT-PCR assay integrity. Minimum bead count quota and control channel responses were used to verify the integrity of the detection channel responses which minimizes the likelihood of false positives and false-negative results caused by operator error, instrument malfunction, non-specific hybridization, or PCR inhibition. The responses of all detection channels are checked against pre-established cut-offs then identified as mRT-PCR positive, negative or inconclusive. For each sample, the MFI of each control bead class was checked against a cut-off value. A given result was considered invalid when the MFI value of the IC, NC or FC controls was out of range, or when both the response of the AC was <20 and no detection channels exceeded cut-off. Results were considered valid when the AC was <20 and any detection channel exceeded cut-off. The AC response can be diminished by a strong positive response on a detection channel caused by competition in the RT-PCR reaction. If the responses of all control channels are acceptable, the number of beads counted for control and detection channels are checked (≥40 beads per channel). If a low bead count for any control channel occurred, then all results for that sample were considered invalid. A low bead count on a given detection channel was considered

206 an invalid result for the channel in question.

207 **Data analysis.** Raw data exported from the Bio-Plex instrument were imported into MATLAB
208 (MathWorks) then analyzed using Microsoft Excel. If at least one result from a duplicate sample
209 analysis exceed cut-off, the sample was assigned as mRT-PCR positive. Receiver Operating
210 Characteristic (ROC) plots (24) were generated using a custom MATLAB program. Published
211 guidelines (25) were followed for the calculations of diagnostic test accuracy and statistical methods to
212 quantify uncertainty.

213 **Cut-off values.** The mRT-PCR responses for this assay are typically non-Gaussian and therefore
214 non-parametric methods were used to determine cut-offs. Each channel in the multiplex has a distinct
215 distribution of responses to true negative samples and therefore each is assigned its own cut-off value.
216 For each detection channel of the mRT-PCR assay, the responses to true negative samples were ranked
217 according to magnitude, then cut-off values were identified as the response (MFI value) which gave a
218 false positive rate closest to 5% (without exceeding 5%), corresponding to a diagnostic specificity of at
219 least 95% (3D MFI $\geq$ 6.5, 5´UTR MFI $\geq$ 5.5). Other mRT-PCR detection channels for the FMD look-
220 alike disease viruses had similar cut-off values. For rRT-PCR, the cut-off was Ct $\leq$32 for both 3D and
221 5´UTR assays (34).

222

223 **RESULTS**

224

225 **Clinical sample validation.** Receiver Operating Characteristic (ROC) plots were constructed to
226 compare the diagnostic performance of the 3D and 5´UTR assays in rRT-PCR and mRT-PCR formats
227 (Figure 2). For the purpose of this evaluation, VI with Ag-ELISA served as the reference method and
228 therefore assigned samples as true negative or true positive, with the caveat that rRT-PCR is known to
229 detect FMDV in some samples considered negative by VI with Antigen-ELISA (35). Each plot shows
230 the true positive fraction (TPF; sensitivity) versus the false positive fraction (FPF; 1-specificity) over the

231 entire range of cut-off values. The ROC plots indicate that the 3D and 5´UTR assays in mRT-PCR
232 format lost some ability to distinguish between true negative and true positive samples, as compared to
233 rRT-PCR. The associated areas under each ROC curve also indicate the level of test performance in the
234 absence of a cut-off value. Transfer to the multiplex format had the greatest effect upon the 5´UTR
235 assay where the area under the curve was reduced to 0.773 in comparison to 0.942 for the rRT-PCR
236 format. In contrast, the effect upon the 3D assay was less apparent: 0.955 and 0.985 for the mRT-PCR
237 and rRT-PCR formats respectively. The ROC plots were generated using 3D and 5´UTR assay results
238 independently. In practice, 3D and 5´UTR assay results would be used in combination, an intrinsic
239 feature of the mRT-PCR format. The trade-off between TPF and FPF shown by ROC plots can be used
240 to inform cut-off selection. The cut-off value for all detection channels was determined from the mRT-
241 PCR response to true negative samples (n=74) using a specificity of 95%.

242 Table 2 summarizes the performance metrics of the 3D and 5´UTR assays when used independently
243 or in combination for both formats. Results are presented according to serotype then summarized for all
244 serotypes. In some cases, the rRT-PCR response for true positive samples (3D; n=5, 5´UTR; n=9) and
245 true negative samples (3D; n=5, 5´UTR; n=2) yielded Ct values that were beyond the cut-off. In
246 practice, these samples would be considered weak rRT-PCR positives and retested.

247 Table 3 shows a three-way comparison of mRT-PCR and rRT-PCR against the reference method
248 using the combined 3D and 5´UTR assay results. The agreement between mRT-PCR and rRT-PCR for
249 true positive samples was 95.8% (204/213). Two samples classified as FMDV-negative by VI and Ag-
250 ELISA tested positive by both formats (SYR 6/2002, LAO 16/2003). Similarly, three samples (SYR
251 7/2002, TUR 17/2002, BHU 5/2004) which were negative by VI and Ag-ELISA tested positive by rRT-
252 PCR but were negative by mRT-PCR due to its higher LOD. These findings are consistent with earlier
253 studies (14, 35) where higher analytical sensitivity of rRT-PCR enabled detection of FMDV in samples
254 designated negative by VI and Ag-ELISA. Due to its higher LOD, the mRT-PCR missed 9/209 positive
255 samples detected by rRT-PCR. Four mRT-PCR false positives had MFI responses that were close to the

256 cut-off which was defined using a specificity of 95%. Four mRT-PCR and rRT-PCR false negatives
257 were samples of FMDV serotype A (NIG 12/74), C (PHI 2/89) and O (O_1 Manisa TUR 8/69, YEM
258 1/2001). However, the rRT-PCR did generate Ct responses for two of these samples including PHI 2/89
259 (3D; 38.94, 5´UTR; 38.51) and O_1 Manisa (3D; 32.71). As the Ct values were beyond the cut-off they
260 would be considered weak rRT-PCR positives and retested. These false negatives which generated
261 either weak or no responses in mRT-PCR and rRT-PCR formats were likely caused by mutations in the
262 FMDV gene segments targeted by the 3D and 5´UTR assays. They could also be due to low amounts of
263 virus present, particularly for O_1 Manisa, which is a titrated sample used as a positive control in the
264 diagnostic rRT-PCR that was deliberately set to be only weakly positive.
265 In addition to the 287 samples used to evaluate the mRT-PCR assay, eleven additional suspect FMDV
266 field samples of undetermined serotype were analyzed. These samples were found to be negative by VI
267 and Ag-ELISA but confirmed positive by combined 3D and 5´UTR rRT-PCR during a previous study.
268 These samples were analyzed by the mRT-PCR assay, which detected 11/11 of these samples,
269 demonstrating that the multiplex also detects FMDV in clinical samples that were most likely rendered
270 non-viable for VI between collection and laboratory receipt.
271 **Limit of detection.** The limit of detection (LOD) of the 3D and 5´UTR assays in mRT-PCR and
272 rRT-PCR formats were compared using serially diluted clinical samples of serotype O or SAT 2 (Figure
273 3). At the defined cut-off values, the mRT-PCR LOD was higher than rRT-PCR by approximately 5-
274 625 times for the 3D assay, and 25-125 times for 5´UTR, depending on serotype. The higher LOD of
275 the mRT-PCR therefore caused the loss of diagnostic performance evident in the ROC plots. Further
276 assay optimization efforts are underway to improve the limits of detection of the 3D and 5´UTR assays
277 in the mRT-PCR format.
278 **Differential detection.** Characterization of the diagnostic accuracy of the look-alike disease assays
279 within the mRT-PCR assay is ongoing and will be reported separately. The mRT-PCR assay was
280 positive with three SVDV field samples (ITL 4/77, HKN 1/80, HKN 5/91), two VESV isolates

281 (serotypes -B51 and -H54), San Miguel sea lion virus (serotypes SMSV-7, -9, -10, -11 and -13) and
282 cetacean calicivirus (CCV, Tur-1, dolphin). The mRT-PCR did not detect VESV -B1-34, other VESV
283 serotypes including bovine (Bos-1 (Tillamook)), feline (A4), reptile (rattlesnake), and skunk, and VSV
284 (serotype NJ 15/88 CP211634 and Indiana 1 subtype Ind 2 Maipu Argentina). Feline calicivirus is in a
285 distinct genomic group from the other VESV viruses tested and was not expected to be detected by this
286 assay. mRT-PCR identified look-alike disease viruses in four suspect FMD field samples that had
287 previously been designated FMDV-negative by VI and rRT-PCR (Figure 4). Two samples from cattle
288 (IRN 4/2002, IRQ 58/2002) were mRT-PCR positive for parapoxvirus. The parapox-3 assay generated
289 the strongest response, however all three assays exceeded their respective cut-off values for both
290 samples. Two further cattle samples (UKG 36/94, UKG 37/94) were mRT-PCR positive for BVDV that
291 were also confirmed FMDV-negative by all methods. The mRT-PCR simultaneously detected the co-
292 infection of FMDV and BVDV in a FMDV true positive sample (HUN 2/72). The presence of BVDV,
293 which causes a prevalent disease of cattle, did not mask the detection of FMDV.
294 Cross-talk between detection channels was minimal, even at the relatively high concentrations of
295 FMDV RNA in many of the field samples tested. A matrix of correlation coefficients for all channel
296 pairings was calculated using the results of all FMDV true positive samples (n=213). The correlation
297 coefficients of the 3D and 5´UTR channels with other channels did not exceed 0.209 and 0.114,
298 respectively.
299
300 **DISCUSSION**
301
302 The mRT-PCR assay was able to detect FMDV at clinically relevant concentrations. The apparent
303 concentration of FMDV in the clinical samples was relatively high as might be expected with vesicular
304 epithelial tissue which, when infected, is rich in virus. The performance of mRT-PCR and singleplex
305 rRT-PCRs was compared using VI and Ag-ELISA to define FMDV true positives and true negatives.

306 Although VI and Ag-ELISA are established methods for the detection of FMDV, previous studies (14,
307 35) have demonstrated that rRT-PCR has higher diagnostic sensitivity and can detect virus in additional
308 samples, which for the purposes of this study would be classified as "true" negatives. For the majority
309 of true positive field samples, the 3D mRT-PCR response was saturated and grouped far from the cut-
310 off. The 5´UTR mRT-PCR signal was generally lower and clustered on either side of the cut-off. The
311 higher LOD of both assays in the mRT-PCR format may be caused by low-level non-specific
312 interactions between primer sets that could reduce amplification efficiency. The primers used in the
313 5´UTR assay were not originally designed with multiplexing in mind. In order to recognize a wide range
314 of FMDV isolates, the 5'UTR primers have a high degree of degeneracy (32-fold and 8-fold for the
315 forward and reverse primers respectively) that could increase the likelihood of non-specific interactions
316 with other primer sets in the multiplexed reaction mixture. Whilst primer probe sets incorporating
317 degenerate nucleotides can offer broader coverage of highly-variable gene segments, further work is
318 required to refine their design for multiplexed assays. Asymmetric PCR and multivariate optimization
319 may lead to further improvements in the LODs of the 3D and 5´UTR mRT-PCR assays.
320 The 3D and 5´UTR assay responses had serotypic bias, a finding which agrees with earlier
321 observations (4, 14). In mRT-PCR format, the 3D assay was less sensitive for A and C serotypes
322 whereas the 5´UTR was less effective against SAT serotypes. For all serotypes collectively, the 3D
323 assay was more sensitive than the 5´UTR within each format (rRT-PCR 3D 96.7% vs. 5´UTR 87.3%,
324 mRT-PCR 3D 90.1% vs. 5´UTR 58.7%). In an earlier evaluation of the rRT-PCR assays (14), the
325 diagnostic sensitivity of the 3D (97.7 %) was found to be slightly higher than the 5´UTR assay (95.4%).
326 The diagnostic sensitivity increased when the results of the 3D and 5´UTR assays were used in
327 combination. This is due to the "or" nature of the combination, whereby a single mRT-PCR positive
328 result on the 3D or 5´UTR channel generates a combined mRT-PCR positive result. The diagnostic
329 sensitivity of the combined mRT-PCR assay was 93.9%, compared to 98.1% for combined rRT-PCR.
330 The loss of diagnostic sensitivity from rRT-PCR to mRT-PCR, due to higher limits of detection, was

331 partially offset by the inherent ability of the multiplex assay to screen multiple loci simultaneously.
332 Combined rRT-PCR only increased the diagnostic sensitivity by 1.4% compared to using the 3D rRT-
333 PCR assay in isolation which could factor into the cost-benefit of conducting parallel assays. As the
334 mRT-PCR is a screening assay that would most likely be used in conjunction with confirmatory tests,
335 potential users may be more tolerant of lower specificity in order to achieve higher sensitivity. The
336 negative predictive value was also higher for combined assays, because of the "or" nature of the
337 combined result as false negatives only occurred when both the 3D and 5´UTR results agreed.
338 The differential detection of FMDV from look-alike disease viruses, which included ssRNA and
339 dsDNA targets, was demonstrated by testing representative isolates of SVDV, VESV and VSV. For a
340 diagnostic laboratory, this could produce time and cost savings, when compared to testing for each
341 disease using singleplex rRT-PCR assays. For veterinarians, the mRT-PCR assay could increase
342 confidence in a sample identified as FMDV-negative by simultaneously screening for the presence of
343 look-alike diseases. For networks of veterinary diagnostic laboratories, an mRT-PCR assay could
344 facilitate embedded foreign animal disease surveillance whilst conducting routine testing of endemic
345 animal disease viruses. An inter-laboratory evaluation of this multiplex assay was recently conducted in
346 fourteen US National Animal Health Laboratory Network laboratories; the results suggested the mRT-
347 PCR technology could be operated successfully in this setting. The diagnostic performance evaluation
348 for the look-alike disease assays in the mRT-PCR format is underway and will be reported separately.
349 The mRT-PCR format is compatible with procedures and instrumentation used for rRT-PCR. The use
350 of a single method to prepare clinical samples for mRT-PCR and rRT-PCR analysis was demonstrated
351 in this study. The mRT-PCR requires the post-processing of RT-PCR product to the microsphere array
352 which takes ~50 min per 96-well plate using manually operated multi-channel pipettes. The 96-well
353 plate format provides convenient interchangeability between manual and automated platforms. With this
354 reagent set, the Bio-Plex flow cytometer analyzed each well in ~40 s, or ~1 h per 96-well plate.
355 Although the diagnostic sensitivity of the mRT-PCR for FMDV detection is slightly lower than

singleplex rRT-PCR, it provides significantly more diagnostic information. With 17 detection channels for seven different viruses, the current prototype panel generates 1632 individual assay results per 96-well plate. The microsphere suspension array is a versatile platform compatible with many different types of diagnostic tests, including immunological and serological assays which could increase its utility within a veterinary diagnostic laboratory, not only for outbreak response and recovery but also for routine testing for endemic diseases. The inherent flexibility of the Luminex array also enables the composition of a given multiplex assay to be altered by simply adding or removing detection channels. Refinements to the first version of the mRT-PCR assay described herein are currently underway, including the development of two species-specific panels for more comprehensive coverage. These new panels incorporate additional assays for other FMDV look-alike diseases. The bovine-specific panel incorporates assays for FMD, Malignant Catarrhal Fever (MCF), Rinderpest, Bluetongue, BHV-1, BVD, Parapox and VS. The porcine-specific panel includes assays for FMD, SVD, VES, VS and Porcine Reproductive and Respiratory Syndrome (PRRS).

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FIGURE CAPTIONS

FIG. 1. Schematic of the multiplex RT-PCR assay. In the presence of target nucleic acid, the biotinylated forward primer is extended during the PCR. The PCR product is hybridized to the microsphere array, whereby the extended forward primer binds to the complementary probe-labeled microsphere. The complex is labeled with fluorescent reporter (SAPE) then analyzed using a Bio-Plex flow cytometer. The fluorescence (570 nm) of bound reporter molecules is measured, and the median fluorescence intensity is calculated for each microsphere class, then compared to a cut-off to indicate the presence or absence of a target nucleic acid sequence in a sample.

FIG. 2. Receiver Operating Characteristic (ROC) plots for the RNA polymerase (3D) and the 5´untranslated region (5´UTR) assays in singleplex real-time reverse transcriptase PCR (rRT-PCR) and multiplexed reverse transcriptase polymerase chain reaction (mRT-PCR) formats. True positive samples (*n*=213) representative of all FMDV serotypes and true negative samples (*n*=74) were analyzed. The area under the curves are; 3D rRT-PCR (0.985), 5´UTR rRT-PCR (0.942), 3D mRT-PCR (0.955) and 5´UTR mRT-PCR (0.773).

FIG. 3. Comparison of the RNA polymerase (3D) and 5´untranslated region (5´UTR) assay responses in multiplex RT-PCR and singleplex rRT-PCR formats by consecutive 5-fold serial dilutions of three different FMDV true positive epithelial suspension samples (undiluted to more dilute from right to left). The multiplex RT-PCR detection was less sensitive than singleplex rRT-PCR (approximately 5-625 times and 25-125 times, respectively for 3D and 5´UTR signatures). Cut-off values for rRT-PCR ($Ct \leq 32$) and mRT-PCR (3D MFI ≥ 6.5, 5´UTR ≥ 5.5) are indicated by the vertical and horizontal dashed lines, respectively. The 5´UTR signature did not respond to the SAT 2 (SAU 4/2000) sample at any

522 dilution in singleplex or multiplex formats and was omitted from the plot. Error bars indicate ±1σ of the
523 mean (n=2) response from the signature in mRT-PCR format. For rRT-PCR each sample was analyzed
524 in singlet (n=1).

525

526 **FIG. 4.** Multiplex RT-PCR assay identification of FMDV look-alike disease viruses in suspect FMDV
527 clinical sample submissions. The mRT-PCR assay ruled out FMDV whilst simultaneously ruling in
528 FMD look-alike disease viruses. A and B show the multi-loci detection of parapox viruses. C shows
529 BVDV detection in field samples from cattle (UKG 36/94, UKG 37/94) that were tested FMDV-
530 negative by virus isolation and rRT-PCR and the simultaneous detection of FMDV and BVDV (HUN
531 2/72). mRT-PCR cut-offs indicated by the vertical dashed lines were PPOX 1 (≥ 7.5), PPOX 3 (≥ 9.5)
532 and BVDV (≥ 6.5). The horizontal dashed lines indicate the cut-off for FMDV 3D (≥ 6.5).

533 **TABLE 1.** Primer and probe sequences of the multiplex RT-PCR assay.

Assay name	Forward Primer (5´→3´) Reverse Primer (5´→3´)	Probe (5´→3´)
BHV 1	GŦGCCAGCCGCGŦAAAAG GACGACTCCGGGCTCTTTT	TCCTGGTTCCAGAGCGCTAACATGGAG
BHV 2	TGAGGCCŦATGTATGGGCAGŦT GCGCGCCAAACATAAGTAAA	AAATAACACGGTGTGCACTTAAATAAGATTCGCG
BTV 1	GCACCCŦATATGTTŦCCAGACCA CAGCTAACTCTTCAGCCACACG	CTAACTCGTGGGCCAATCATCATCTTCTGT
BTV 2	AGAATŦCAGGAŦGGGCAGGA GCACAATTCCCATCCCCTTA	CCATCACACCATTATACTGTACCCGCGTAGC
BVDV	GGTAGTCGŦCAGTGGTŦCGAC CATGTGCCATGTACAGCAGAGAT	CCTCGTCCACGTGGCATCTCGAG
FMDV 3D	ACTGGGŦTTTACAAACCŦGTGA GCGAGTCCTGCCACGGA	GTCCCACGGCGTGCAAAGGA
FMDV 5´UTR	CACYTYAAGRŦGACAYTGRTACŦGGTAC CAGATYCCRAGTGWCICITGTTA	CCTCGGGGTACCTGAAGGGCATCC
PPOX 1	GCAGAŦGCGCTCCŦGGTT GCACCTCTGCTGCTGCAA	CCGACTCCGACGTGGAGAACGTG
PPOX 2	GATGGCCGŦGCAGCŦCTT CGTACAAGATCACGGCCAACT	TGTACGGGCTCATGGGCTTCCG
PPOX 3	GCAGCAGŦGCACCACGŦAGT CGCTGAACCCGTACATCCT	GACTTCGAGGCGGACAACAAGCG
SVDV 1	CAGGAŦAATTTCTŦCCAAGGGC ACGTGAACATTTCGAGCTTCC	TGCATTGTGTCTGATGGTACAACTTGTGACG
SVDV 2	GACTTGŦTGTGGCŦGGAGGA CAGCGCCATGGTGAGGTAG	TGACCGTAATGAGGTCATCGTGATTTCTCAC
SVDV 3	GACAAAGŦGGCCAAGGGAAA CACGTAAACCACACTGGGCT	CTGGCGTCATAGCCTGAATAGTCAAACGCTA
VESV 1	GCCTŦCTCCCTŦCCCAAAA TGAAGGAATGGTTCCGTCAGT	CATCATCGTTGATAACCTTAGATGTGCAATTTGG
VESV 2	GGGAAŦGAGGTGTGCAŦCATT CACGTCTTGATGTTGGCTTGAC	AAATTGGCATAATCAACCTTGTCAGATGAGTCG
VESV 3	GGTCGCŦCTCACTGATGAŦGAGTA GGTGTTATCAGCACCCATTGC	GCTCGGTGCCTGAGTTGGAGGAAG
VESV 4	ACCACCŦCTGGAAACATCŦATGG TTTGTGCACGTGTCACGAAT	CGGGACGGGCATTTGTCACCA
FC	N/A	CAAAGŦGGGAGACGTCGŦTG
IC	N/A	CAAAGTGGGAGACGTCGTTG-Cy3
NC	N/A	CAAAGTGGGAGACGTCGTTG

534

535 BHV = bovine herpes virus-1, PPOX = parapox virus complex, FMDV = foot-and-mouth disease virus,
536 BVDV = Bovine Viral Diarrhea Virus, BTV = bluetongue virus, SVDV = swine vesicular disease virus,
537 VESV = vesicular exanthema of swine virus, NC = negative control, FC = fluorescence control, IC =
538 instrument control, Cy3 = fluorescent cyanine dye, Y = pyrimidines (C/T), R = purines (A/G), W =
539 weak 2-bonds (A/T), I = inosine (universal base). Ŧ = an internal biotinylated dT. All forward primers
540 also include biotinylation at the 5´ terminus. All probes contain an amine attached to the 5´ terminus
541 with a carbon 6 and internal spacer 18. N/A = not applicable.

542

543 **TABLE 2.** Performance metrics for the 3D and 5´UTR FMDV assays in rRT PCR and mRT-PCR

544 formats using independent or combined results.

Performance metric		rRT-PCR			mRT-PCR		
		3D	5´UTR	Combined	3D	5´UTR	Combined
Sensitivity (%)	A	93.5	87.0	97.8	80.4	76.1	93.5
(Fraction)		(43/46)	(40/46)	(45/46)	(37/46)	(35/46)	(43/46)
	Asia 1	100	100	100	100.0	100	100
		(10/10)	(10/10)	(10/10)	(10/10)	(10/10)	(10/10)
	C	95.0	95.0	95.0	85.0	55.0	90.0
		(19/20)	(19/20)	(19/20)	(17/20)	(11/20)	(18/20)
	O	96.7	86.7	97.8	91.1	70.0	92.2
		(87/90)	(78/90)	(88/90)	(82/90)	(63/90)	(83/90)
	SAT 1	100.0	88.9	100	100	16.7	100
		(18/18)	(16/18)	(18/18)	(18/18)	(3/18)	(18/18)
	SAT 2	100.0	71.4	100	95.2	9.5	95.2
		(21/21)	(15/21)	(21/21)	(20/21)	(2/21)	(20/21)
	SAT 3	100.0	100	100	100	12.5	100
		(8/8)	(8/8)	(8/8)	(8/8)	(1/8)	(8/8)
	All	96.7	87.3	98.1	90.1	58.7	93.9
		(206/213)	(186/213)	(209/213)	(192/213)	(125/213)	(200/213)
C.I. (95%)		93.4-98.4	82.2-91.1	95.3-99.3	85.4-93.5	52-65.1	89.8-96.4
Specificity (%)		94.6	95.9	93.2	93.2	94.6	91.9
C.I. (95%)		86.9-97.9	88.7-98.6	85.1-97.1	85.1-97.1	86.9-97.9	83.4-96.2
Sample Disease Prevalence (%)		74.2	74.2	74.2	74.2	74.2	74.2
PPV (%)		98.1	98.4	97.7	97.5	96.9	97.1
NPV(%)		90.9	72.4	94.5	76.7	44.3	84.0
Efficiency (%)		96.2	89.5	96.9	90.9	67.9	93.4

545 Sensitivity is the fraction of the 213 true positive epithelial tissue samples (subjected to both multiplex

546 RT-PCR and rRT-PCR assays) that are assigned as FMDV-positive by the indicated assay. C.I.
547 Confidence Interval, PPV Positive Predictive Value, NPV Negative Predictive Value.

548

549 **TABLE 3.** 3-Way comparison table for the combined results of 3D and 5´UTR assays in rRT-PCR and
550 mRT-PCR formats.

Method Result		Total Samples	True Diagnosis	
Combined rRT-PCR	Combined mRT-PCR		Positive	Negative
Positive	Positive	202	200	2
Positive	Negative	12	9	3
Negative	Positive	4	0	4
Negative	Negative	69	4	65
Total		287	213	74

551

FIG.1

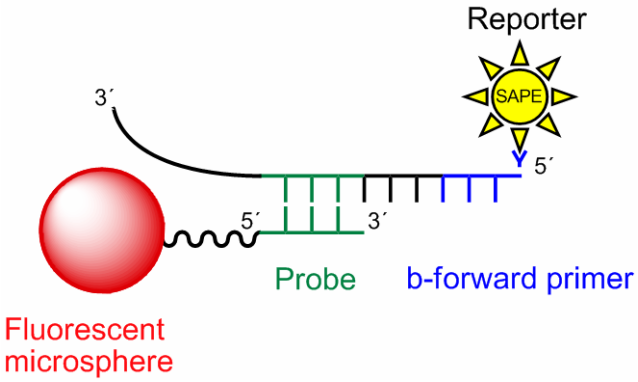

Reporter
SAPE
3´
5´
5´
3´
Probe
b-forward primer
Fluorescent
microsphere

FIG.2

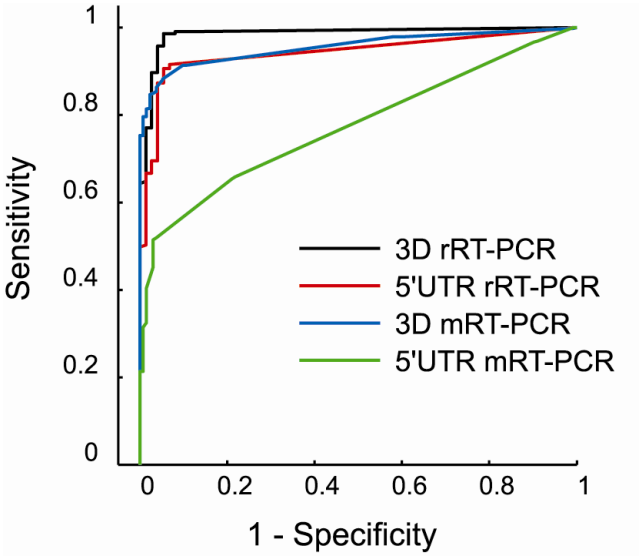
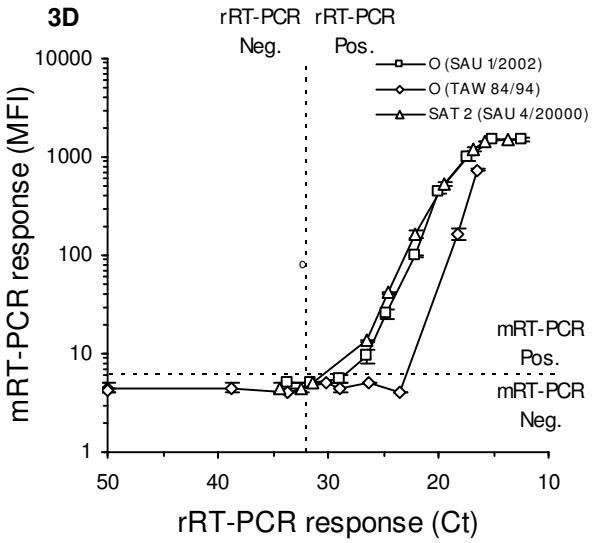

Sensitivity
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
1 - Specificity
3D rRT-PCR
5'UTR rRT-PCR
3D mRT-PCR
5'UTR mRT-PCR

FIG. 3


3D
rRT-PCR Neg.
rRT-PCR Pos.
O (SAU 1/2002)
O (TAW 84/94)
SAT 2 (SAU 4/20000)
mRT-PCR Pos.
mRT-PCR Neg.
mRT-PCR response (MFI)
rRT-PCR response (Ct)
10000
1000
100
10
1
50
40
30
20
10

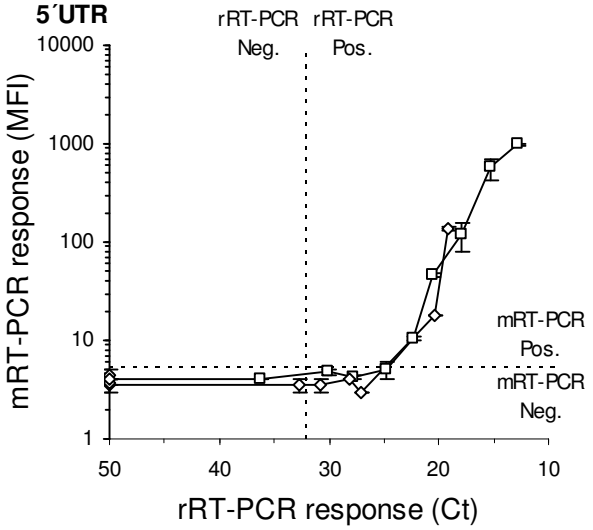
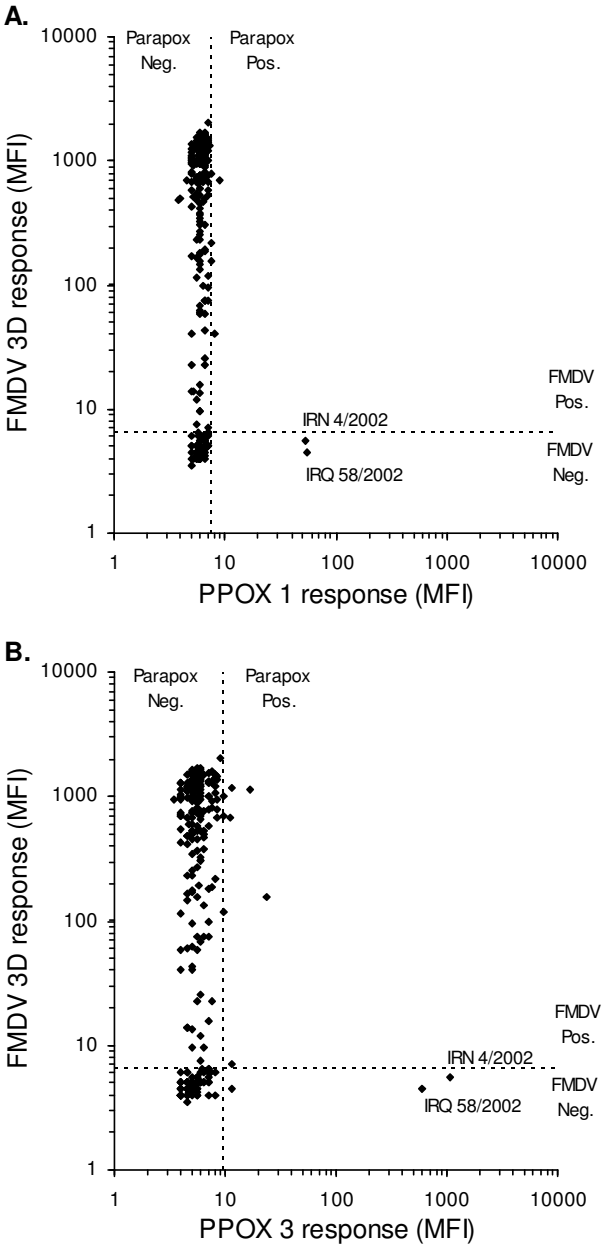
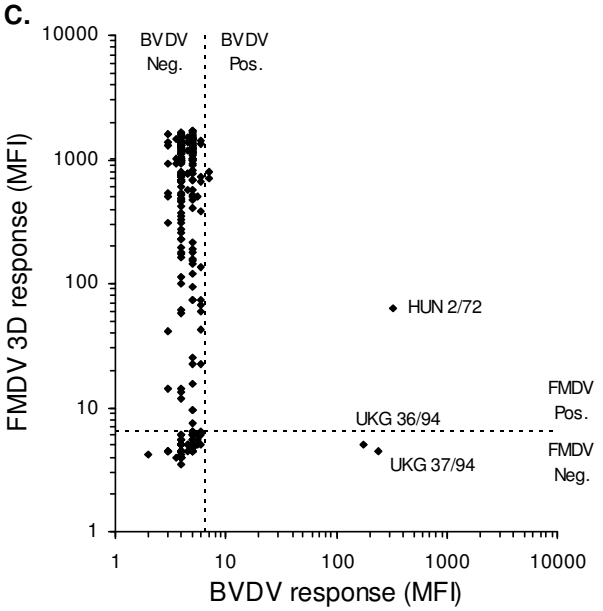

5´UTR
rRT-PCR Neg.
rRT-PCR Pos.
mRT-PCR Pos.
mRT-PCR Neg.
mRT-PCR response (MFI)
rRT-PCR response (Ct)
10000
1000
100
10
1
50
40
30
20
10

FIG. 4


A.
Parapox Neg.
Parapox Pos.
FMDV 3D response (MFI)
PPOX 1 response (MFI)
IRN 4/2002
IRQ 58/2002
FMDV Pos.
FMDV Neg.
10000
1000
100
10
1
B.
Parapox Neg.
Parapox Pos.
FMDV 3D response (MFI)
PPOX 3 response (MFI)
IRN 4/2002
IRQ 58/2002
FMDV Pos.
FMDV Neg.

C.


10000
1000
100
10
1
FMDV 3D response (MFI)
BVDV
Neg.
BVDV
Pos.
HUN 2/72
FMDV
Pos.
UKG 36/94
FMDV
Neg.
UKG 37/94
1
10
100
1000
10000
BVDV response (MFI)