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Further development of a reverse-transcription loop-mediated isothermal amplification (RT-LAMP) assay for the detection of foot-and-mouth disease virus and validation in the field with use of an internal positive control

Short running title: RT-LAMP for FMDV in the field with an IPC

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Summary

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hooved animals. Global outbreaks have highlighted the significant economic, trade, psychosocial and animal welfare impacts that can arise from the detection of disease in previously 'FMD-free' countries. Rapid and early diagnosis provides significant advantages in disease control and minimisation of deleterious consequences. We describe the process of further development and validation of a reverse-transcription loop-mediated isothermal amplification foot-and-mouth disease virus (RT-LAMP-FMDV) test, using a published LAMP primer set, for use in the field. An internal positive control (IPC) was designed and introduced for use with the assay to mitigate any intrinsic interference from the unextracted field samples and avoid false negatives. Further modifications were included to improve the speed and operability of the test, for use by non-laboratory trained staff operating under field conditions, with shelf-stable reaction kits which require a minimum of liquid handling skills. Comparison of the assay performance with an established laboratory based real-time reverse transcriptase PCR (rRT-PCR) test targeting the 3D region of FMD virus (Tetracore Inc, USA) was investigated. LAMP has the potential to complement current laboratory diagnostics, such as rRT-PCR, as a preliminary tool in the investigation of FMD. We describe a strategic approach to validation of the test for use in the field using extracted RNA samples of various serotypes from Thailand and then finally unextracted field samples collected from FMD suspected animals (primarily oral lesion swabs) from Bhutan and Australia. The statistical approach to validation was performed by Frequentist and Bayesian latent class methods, which both confirmed this new RT-LAMP-FMDV test as fit-for-purpose as a herd diagnostic tool with diagnostic specificity >99% and sensitivity 79% (95% Bayesian credible interval: 65, 90%) on unextracted field samples (oral swabs).

Keywords

cattle; detection and validation; foot-and-mouth disease virus; in-field test; internal positive control; RT-LAMP

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1 Introduction

Foot-and-mouth disease virus (FMDV) is a single-stranded RNA virus which belongs to the genus *Aphthovirus* within the *Picornaviridae* family and has seven serotypes which have varying dominance in different parts of the world – O, A, C, Asia 1, SAT1, SAT2 and SAT3. It causes a highly contagious disease affecting both domesticated and wild cloven-hooved animals. Although it is a disease of low mortality, its high infectivity and the resulting drop in livestock productivity make foot-and-mouth disease (FMD) one of the most significant diseases in global animal production and trade.

Outbreaks in 'disease-free' countries can result in substantial economic losses, through trade restrictions and costs associated with responding to outbreaks, eradication and regaining recognition of 'free-status' by the members of the World Organisation for Animal Health (OIE), that has been estimated at US$ 1.5 billion a year (Knight-Jones & Rushton, 2013). This was evident in the UK outbreak in 2001 which resulted in costs of over US$ 9.2 billion and the slaughter of over six million animals for disease control purposes and welfare reasons (FAO, 2002). Simulated estimates for the median costs of an outbreak in the US are up to US$ 69 billion, based on a delay of up to 22 days for diagnosis (Carpenter et al., 2011), while in Australia a large multi-jurisdiction FMD outbreak is predicted to cost AU$ 52 billion over a ten-year period (Buetre et al., 2013).

In the event of an outbreak, rapid and accurate diagnosis of the disease is imperative, particularly in countries where the submission of diagnostic samples from remote areas to centralised laboratories can delay implementation of disease control measures and lead to ongoing spread and escalating costs. A case study of a simulated outbreak in the US estimated that for each hour FMD detection is delayed beyond 21 days, can result in an additional 2000 animals being slaughtered and further economic losses of over US$ 500 million (Carpenter et al., 2011). This highlights the critical nature of a timely and accurate confirmation of FMD and a simple, rapid and reliable in-field diagnostic tool to support decision-makers in this process.

Currently molecular diagnosis is determined by the OIE recommended real-time RT-PCR (rRT-PCR) assays (Callahan et al., 2002; OIE, 2018a; Reid et al., 2002; Shaw et al., 2007) which relies on samples being transported to a central diagnostic laboratory for processing by a trained technician - a lengthy procedure which can delay crucial decision making. To reduce time delays the rRT-PCR assay has previously been used in the field, mainly on specialised equipment which performs both the extraction and amplification of the virus (Callahan et al., 2002; Howson, Armson, et al., 2017) but can now also be done on a smaller portable platform and directly on unextracted clinical samples, with an assay that takes about an hour to perform on the device (Howson et al., 2018).

Isothermal technologies are emerging as a popular alternative by exploiting the use of enzymes at a constant temperature to amplify genome segments, often much quicker than PCR, using relatively simple equipment. One such method, loop-mediated isothermal amplification (LAMP), is gaining

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momentum as a popular diagnostic tool and has been used to develop assays for the detection of a number of animal diseases including FMD (Best et al., 2018; Mansour et al., 2015). The original FMD RT-LAMP was designed to target a section of the conserved 3D RNA polymerase gene, allowing it to detect all serotypes of FMD with a sensitivity that is equivalent to rRT-PCR (Dukes et al., 2006). This pan-serotypic FMD RT-LAMP assay has been further adapted for use in the field, initially being combined with a lateral flow device to detect FMDV in unextracted epithelial suspensions by simply diluting the sample (Waters et al., 2014) and then with lyophilised reagents and portable equipment in the field in Africa (Howson, Armson, et al., 2017). Despite several other LAMP assays being developed for FMD, including those for serotyping FMDV (Chen et al., 2011a; Madhanmohan et al., 2013), their limitations include an inability to amplify or lack of testing on all seven serotypes (Chen et al., 2011b; Lim et al., 2018; Maryam et al., 2017; Ranjan et al., 2014; Shao et al., 2010) or a lengthy testing procedure that is not as practical for in-field testing (Guan et al., 2013).

Here we report the validation of the pan-serotypic FMD RT-LAMP assay (Dukes et al., 2006) that has been further adapted for use with unextracted and undiluted samples that are added directly into the reaction. The samples can be assayed in the field in conjunction with an internal positive control (IPC) which has been designed and included into the system to avoid false negative results that may arise due to interference of intrinsically "dirty" unextracted field samples. We aimed to establish an assay that was "fit for purpose" and able to be easily deployed by non-laboratory trained staff for initial in-field testing of FMD in the investigation of suspect FMD cases.

2 Materials and Methods

2.1 RNA and field samples used for RT-LAMP evaluation

Samples (n=271) representative of three different geographical areas were used for development and validation of the RT-LAMP assay. They include:

(1) Total RNA extracted from 51 different reference samples, representing serotypes O, A and Asia1 (kindly provided by the Regional Reference Laboratory for Foot and Mouth Disease in the South East Asia, Pak Chong, Thailand) (Table 1), with the corresponding C_T values obtained from the rRT-PCR test targeting the 3D region of FMD virus genome (Tetracore assay; Callahan et al., 2002). RT-LAMP assay was performed using 2µl of total RNA per reaction, and the results were compared with the Tetracore assay;

(2) Australia is designated to be historically free of FMD and to estimate diagnostic specificity (DSp), clinical samples collected from Australian animals from the state of Victoria that showed clinically relevant differential diagnostic symptoms were tested for FMD exclusion. These samples included oral or foot swabs collected from cattle (n=96) and sheep (n=90) subsequently diagnosed with ovine footrot, bovine papular stomatitis (parapox) and Upper Alimentary Ulcerative Syndrome (UAUS).

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Samples comprised swabs collected by veterinarians during clinical disease investigations between 2014 and 2018, sent to the Veterinary Diagnostic laboratory (Agriculture Victoria) at AgriBio, Melbourne, Australia in either viral transport media (VTM - brain-heart infusion broth containing 50U/L penicillin, 5mg/ml streptomycin, 1mg/ml gentamicin, 1mg/ml kanamycin and 0.5U/L mycostatin) or phosphate buffered saline (PBS, 20mM EDTA, pH 8) for diagnosis, and stored at -80°C; (3) FMDV field samples consisting of oral lesion swabs (n=10) and foot lesion swabs (n=3) from FMD-suspected cattle in Bhutan, collected in October 2018, and archival swab samples (n=21) collected by the National Centre for Animal Health (Thimphu, Bhutan) between June 2017 to September 2018 (Table 2). All samples were taken by veterinarians as part of routine diagnosis. The swabs collected in October 2018 were placed in PBS, pH 7.4 (Sigma-Aldrich Pty. Ltd., Macquarie Park, Australia) on ice and later stored at -20°C until further use. Archived samples were shipped to the World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD) in Pirbright, United Kingdom, for further confirmation of FMDV, where they were typed as serotype O.

Bhutanese and Australian swab samples were tested directly in the RT-LAMP assay by transferring 1µl of the unextracted medium in which the swab had been stored, into the reaction using a disposable inoculating loop. Medium (50µl) from these samples were also RNA extracted using QIAamp viral RNA mini Kit (Qiagen, Hilden, Germany)(Bhutan) or the MagMAX-96 Viral Isolation kit (Life Technologies, Carlsbad, USA) in a Kingfisher Flex Purification System (Thermo Fisher Scientific, Waltham, USA)(Australia) following manufacturers' instructions, for confirmatory laboratory testing in the Tetracore rRT-PCR assay.

2.2 Preparation of RNA standard

A synthetic 173bp dsDNA gBlocks® Gene Fragment (Integrated DNA Technologies, Inc., Coralville, USA), that covers the LAMP target region in the 3D gene of FMDV type O strain UKG/35/2001 (GenBank entry AJ539141, bases 7850-8022, Figure 1) was amplified in a PCR using the PCRBIO HiFi kit (PCR Biosystems Ltd., London, UK). A forward primer combining the FMDV F3 LAMP primer sequence with a T7 promoter sequence at the 5' end (5'-TAATACGACTCACTATAGGGAG-CATGGACTATGGAACTGGGT-3') and the FMDV B3 LAMP reverse primer produced an amplicon with a T7 promoter for RNA transcription. The PCR reaction (50µl) consisted of 10µM of each primer and the following cycling conditions were used: 95°C, 5min, 40 cycles of 95°C for 30s, 55°C for 30s, 72°C for 20s, and a further 2min at 72°C. A 195bp amplicon was produced and used as template for *in vitro* transcription with the MEGAscript T7 transcription kit (Life Technologies) to produce a 178nt synthetic viral RNA transcript. DNA was removed with the TURBO DNA-free kit (Life Technologies). The RNA concentration was quantified with the Qubit RNA HS assay kit (Life Technologies) and the copy number of the target region determined with a DNA/RNA Copy Number Calculator

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(http://endmemo.com/bio/dnacopynum.php). The RNA standard was used as template to determine the limit of detection (LOD) of the RT-LAMP assay, and as a synthetic virus positive control in all RT-LAMP assays.

2.3 Designing the Internal Positive Control (IPC)

An IPC was developed to ensure the reliability of the LAMP assay and to control for false negative results. The IPC is nearly identical in sequence to the FMDV RNA standard, ensuring the FMDV LAMP primers bind and amplify the IPC in the same manner. To ensure the IPC is easily distinguished in the assay, a ten nucleotide region of sequence between the F1 and B1 primer binding regions of the FMDV target sequence (GGACCATACA) (Figure 1) was altered with nucleotide changes and the addition of ten nucleotides which resulted in (ATGAACATATAGTATCAATT) the overall GC content of the target region being lowered from 57% to 53%, thereby reducing the annealing temperature by 1.2°C according to the Oligo Calc: Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/ OligoCalc.html) (Kibbe, 2007). The IPC was constructed and prepared according to the method described above for the FMDV RNA standard, resulting in a 188nt RNA transcript.

The IPC was designed to be competitive by amplifying with the same primers as the FMDV target. The optimal amount of IPC to be added to the LAMP reaction was determined via serial dilutions with four separate master mixes as previously described (Cook et al., 2013), to find the highest dilution that is consistently amplified in the absence of the target nucleic acid.

2.4 RT-LAMP assay

The assay was performed using previously published primers (Dukes et al., 2006). Reactions consisted of a lyophilised commercial master mix ISO-DR004-RT300 (OptiGene Ltd., Horsham, UK), with primer concentrations, F3/B3 0.2µM, FIP/BIP 2µM and loop primers at 1µM in a total volume of 25µl. All primers were synthesised by Sigma-Aldrich (Castle Hill, Australia) with standard desalting purification.

Amplification occurred at 65°C for 20 min on the portable real-time fluorometer Genie III® (OptiGene Ltd.) instrument, with an annealing step from 98°C to 80°C, at a rate of 0.05°C/sec to distinguish the amplification products. In addition to the samples each run included a negative and positive control (~$1x10^6$ copies of RNA standard).

The Genie III® instrument reports the peak amplification time of a sample, which will be reported here as time to positive (T_P) (minutes: seconds), and the anneal derivative temperature (T_a) (°C). All RT-LAMP analysis was performed using Genie® Explorer v2.0.6.3 software and the default thresholds (OptiGene Ltd.). Samples were called positive for FMDV if an amplified product had a T_a of 87.9°C to

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89.9°C. The mean T_a of 88 tested viral samples was 88.9°C, ±0.9°C. Negatives were confirmed when only the IPC successfully amplified, indicating no inhibition. The IPC had a mean T_a of 87.3°C when added to virus samples.

2.5 Real-time RT-PCR

Real-time RT-PCR Tetracore assay was performed as per Callahan et al. (2002) using the AgPath-ID One-Step RT-PCR Kit (Thermo Fisher Scientific) and 5µl of extracted RNA. The assay was performed on the QuantStudio 5 Real-time PCR system (Thermo Fisher Scientific) for FMD samples (Bhutan), or on the Applied Biosystems 7500 Fast real-time PCR instrument (Thermo Fisher Scientific) in the analytical sensitivity assay and for DSp (Australia), with cycling conditions that consisted of a 10 min RT step at 45°C, followed by 10 min at 95°C, and a 45-cycle PCR (95°C for 15 sec and 60°C for 45 sec).

2.6 Analytical sensitivity and specificity

To estimate the limit of detection (LOD) of the RT-LAMP assay, the FMDV RNA standard was 10-fold serially diluted in Buffer EB (Qiagen) and 5µl was used in each RT-LAMP assay to achieve a final copy number range of 10^6 to 10^0 copies, tested in triplicate, to determine a conservative estimate of the lower LOD. As the target areas of the RT-LAMP and the Tetracore rRT-PCR assays overlap (Figure 1) the analytical sensitivity of the two assays was compared by also testing the RNA standard dilutions, in triplicate, in the rRT-PCR.

To further investigate the LOD in the target matrix, and to replicate the assay in the field where 1µl loop of matrix is introduced into the reaction, the RNA standard was serially diluted in the matrix PBS (used in Bhutan) or VTM (used for most Australian swab samples - parapox and UAUS samples), and 1µl added to the RT-LAMP reaction, each dilution done in triplicate.

Analytical specificity for the primers were previously described by Duke et al. (2006) and Howson et al. (2017). In addition, in our study, we have performed the analytical specificity with samples from Australian sheep and cattle (n=186) diagnosed with ovine footrot, parapox and UAUS (Table 3), to demonstrate specificity of the assay.

2.7 Evaluation of RT-LAMP assay on field samples

The archived and field-collected Bhutanese FMDV samples were used to estimate the diagnostic sensitivity of the RT-LAMP assay on unextracted samples. The ten field-collected oral swabs, collected from a small dairy farm in the hills near Paro, Bhutan, were initially tested “in the field” (at the edge of the farm) within two hours of collection, to determine the robustness and ease of use of the RT-LAMP assay under field conditions while any further retesting, and the assays for the field foot swabs and

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archived samples were performed back in the laboratory (Thimphu, Bhutan). Dried controls and primers were used (Appendix S1).

Samples that did not amplify in the RT-LAMP assay were repeated with the addition of the IPC – added as a 1µl loop (10^4 copies) – to detect the presence of inhibitors that may prevent amplification. Failure of the IPC to amplify confirms the presence of too many inhibitors in the reaction. Amplification of the IPC confirms that few inhibitors if any are present, and therefore that a lack of FMD amplification in the reaction is due to the virus being either absent from the sample, or at such low concentrations that it is below the detection limit of the assay. Samples that failed to allow the IPC to amplify were diluted 1/50 (Bhutan) or with increasing tenfold dilutions (Australia) with Buffer EB and retested to achieve a final result.

2.8 Repeatability of the RT-LAMP assay

The RNA standard has been used as a positive control in all the FMD RT-LAMP assays, except for nine samples from Thailand where the IPC was initially used. The data from these reactions allow us to evaluate the repeatability of the assay between laboratories in three different countries and with multiple operators, as there was a different main operator in each country. In Thailand 2µl of dried and resuspended control (positive, 3 x 10^6 copies/µl; IPC 5 x 10^4 copies/µl) was added to the reaction with a pipette. The Bhutan positive control was dried and resuspended as 1 x 10^6 copies/µl and added with a 1µl loop. In Australia the positive control was 1 x 10^6 copies/µl and 1µl added with a pipette by a field staff (non-laboratory) operator. The coefficient of variation (CV) was calculated as a measure of the variability in the T_P and the T_A of these controls.

2.9 Bayesian latent class analysis

The minimum required sample size was estimated *a priori* to be 95 samples for estimating diagnostic sensitivity (DSe) and 188 samples for estimating DSp, with 95% confidence and absolute precision of ±2.5%, assuming sensitivity (Se) of the RT-LAMP would be >99% and specificity (Sp) to be 98%, using the R package 'epiR' (Stevenson et al., 2018). Where insufficient reference samples are available for such an analysis then a Bayesian latent class analysis is the OIE recommended approach (Kostoulas et al., 2017; OIE, 2018b), making no assumption of the true disease status of the animals from which the samples are derived.

A Bayesian latent class model (BLCM) based on Branscum et al. (2005) was fitted with the assumption that the two assays were conditionally dependent - both nucleic acid-based tests and detecting the 3D polymerase coding region in the genome (Christensen et al., 2011; Dendukuri & Joseph, 2001); neither test was perfect (Enøe et al., 2000) and the true status of the samples was unknown. The data consisted of the joint results of the Tetracore assay and LAMP assay obtained

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from two populations (1 = Bhutanese cattle, 2 = Australian cattle; Tables 2 and 3) with different prevalence of FMD. The cross-classified counts of joint test results (T1+T2+, T1+T2-, T1-T2+ and T1-T2-) for the Tetracore assay as the reference test (T1) and the LAMP assay (T2) as the comparative test were 26, 7, 0, and 0 (population 1), and 0, 0, 0 and 96 (population 2). Because a BLCM that incorporates conditional dependence between tests is not identifiable, prior information on at least two parameters was needed to ensure model identifiability. Given the Tetracore assay is used extensively at the Australian Animal Health Laboratory (AAHL) and around the world, the DSe and DSp estimates of the Tetracore assay from a previously published study (Goris et al., 2009) were used as prior Beta (a,b) distributions for DSe and DSp. Beta (a,b) priors were estimated using Betabuster 1.0 (https://betabuster.software.informer.com) in the 'epiR' library of the R statistical program (R Core Team, 2017; Stevenson et al., 2018) (see Appendix S2 for R code) with assumptions that the DSe was 95% sure to be >0.85 with mode=0.90 [Beta(99.70, 6.19)], and the DSp estimate was 95% sure to be >0.80 with mode=0.98 [Beta (100, 3.02)]. Flat beta (1,1) priors were assumed for the DSe and DSp of the LAMP assay, given no available prior information. Since FMD is endemic in Bhutan we assumed a uniform prior bounded by (0.5, 1). As Australia is FMD free we considered a zero prevalence of the disease in the population, and as a Beta distribution does not have mass over zero, modelled true prevalence (π) = $\pi^* \times \tau$ with a mixture distribution assuming probability tau (τ) from a Bernoulli distribution and if $\pi = 0$ then the probability $\tau 0$ and the uncertainty about the unknown π^* as $\pi^* \sim$ beta ($a\pi$, $b\pi$) with the assumptions that prevalence was 95% sure to be <0.05 with mode=0.01 (beta(1.88, 88.32)). We also tested two prior values for the unknown prevalence in the two populations: a flat beta (1, 1) prior and a uniform (0, 0.5) prior, with the later indicating that that there was no chance of having prevalence exceeding 50%. Scripts were run using OpenBUGS v3.2.3 (Lunn et al., 2009) with convergence estimates derived using one million iterations of simulation with sampling done every 1,000th iteration until the MC error value was <5% of the standard deviation of the node estimate using three assumed chains as initials or three generated initials (see code in Appendix S2), and discarding 5,000 iterations as burn-in. Convergence was assessed by evaluation of the history, and trace plots and calculation of the Gelman-Rubin statistic diagnostic which compares the within and between chain variability of the three chains that were run. Posterior medians with 95% probability intervals (PI) corresponding to the 2.5th and 97.5th percentiles of the Monte Carlo sample were used to summarise parameter estimates of DSe, DSp and prevalence in the two populations.

3 Results

3.1 Analytical sensitivity of the RT-LAMP assay

The RT-LAMP assay is able to amplify a conserved section of the 3D gene of the FMDV, allowing virus detection. The highest dilution of the RNA standard, that mimics the virus, that was amplified reliably

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in all replicate RT-LAMP reactions contained 1,000 copies (Table 4). RNA standard copy numbers below this limit could occasionally be detected by RT-LAMP, down as far as 100 copies, but not reliably. Assays of dilutions (1:2) below 1,000 copies failed to find a lower LOD (not shown). The rRT-PCR in comparison was able to consistently detect down to 10 copies of the RNA standard, indicating that this LAMP assay is 100-fold less sensitive than the rRT-PCR assay.

The intended target matrix of this assay when performed in the field would be VTM which swab samples are placed in routinely, in Australia, or when not available a buffer such as PBS may be used, as we used in Bhutan. To investigate the effects of these matrixes, LOD assays were performed with the RNA standard diluted in each matrix. VTM gave the same result as above with a LOD of 1,000 copies, while PBS was slightly less sensitive at 10,000 copies (Table S1, Appendix S3).

3.2 Analytical specificity of RT-LAMP assay

The inclusivity of the RT-LAMP assay was evaluated using RNA extracted from 51 reference samples of FMDV that represent the serotypes O, A and Asia 1 (Table 1) which were all successfully amplified.

FMDV was not detected using RT-LAMP or rRT-PCR in any of the samples from Australian animals (n=186) diagnosed with ovine footrot, parapox and UAUS (Table 3), showing no cross-reactivity with these disease pathogens (specificity).

3.3 IPC development

The competitive IPC was designed to assess if there was any sample-derived inhibition in the RT-LAMP reactions when unextracted samples were tested. The IPC was successfully amplified and produced an amplicon with a T_a of mean 87.2°C (± 0.2°C), which was easily distinguishable from the viral target or the positive control (88.9°C). The optimal number of copies of the IPC that will consistently amplify from separately made master-mixes was determined as 1,000 copies, which amplified at a mean of 11 min: 17 sec (± 2 min: 41 sec) (data not shown).

Of the unextracted Australian samples tested, 22 (11.8%) exhibited inhibition on initial LAMP testing as demonstrated by the lack of IPC amplification (Table 5). Diluting samples eliminated the inhibition and IPC amplification was detected when retested, producing valid results. Dilutions up to 1/100 were able to restore amplification of the IPC in 95% of these samples.

3.4 Detection of FMD virus in field samples (unextracted swab samples)

The RT-LAMP assay appears to be robust in its assay conditions and as a pen-side test to detect FMD infected animals from various samples in the field (Appendix S1 and Table 2). Out of 33 predominately

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oral swabs from cattle, confirmed to be infected by FMDV by rRT-PCR, 27 tested positive in the RT-LAMP assay as unextracted samples (Tables 2 and 3). The RT-LAMP assay is estimated to have a relative DSe of 82% in cattle when using unextracted samples (Table 3). A closer examination of the results revealed that all those samples that returned a negative result had a rRT-PCR C_T value of >30.

Of the 34 archived samples from Bhutan, nine had to be diluted to confirm a result (Table 2). The IPC successfully indicated that inhibitors were present and preventing amplification of virus in five of these samples. The remaining four samples gave a T_a temperature different than was expected (for the virus), but dilution of the samples brought the T_a back into the expected range, the dilution presumably altering the salt concentration or pH which was the cause of the different T_a.

3.5 Repeatability of the RT-LAMP assay

The repeatability of the FMD LAMP assay was investigated by looking at the variability of the positive control data produced from work done in three countries with more than three operators. The variability of T_P as expressed by the CV was low revealing that the positive control, and hence the FMD RT-LAMP assay, has satisfactory repeatability (Table 6). The coefficient of variation for the T_a remains very low in all countries.

3.6 Bayesian latent class analysis

Outputs of the latent class analysis, that does not make any assumption about the true disease status of the samples and is preferred when there are insufficient reference samples available, are presented in Table 7. The DSe of the RT-LAMP was estimated to be 79% with Bayesian credible intervals encompassing the point estimate derived based on an assumption of sufficient reference samples (Table 3). The DSp of the RT-LAMP inferred to be higher than that of the Tetracore rRT-PCR assay.

4 Discussion

Since the first report of LAMP technology (Notomi et al., 2000), there have been many assays developed for pathogens in the veterinary field, but despite LAMP assays being ideally suited for in-field use, few assays are specifically designed for that exact purpose and are mostly run on extracted samples in laboratories. A FMDV targeted RT-LAMP assay was originally developed to detect the virus in extracted RNA (Dukes et al., 2006), but was later found to work just as efficiently with diluted FMDV infected epithelial, sera or oesophageal-pharyngeal fluid directly added to the assay and using lyophilised kits and portable equipment (Howson, Armson, et al., 2017; Waters et al., 2014), highlighting its potential for in-field use. We have taken this a step further by the use of lyophilised controls, removing the dilution step to streamline assay set up, increasing ease of use for non-

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laboratory personnel, and the inclusion of an IPC to reduce the possibility of false negatives in the field.

The aim of this work was to develop a FMD field assay that can be integrated into routine field investigations of disease to improve diagnostic capability, reduce sample transportation delays and support early decision-making during a disease outbreak. The adoption of purpose-built equipment and shelf stable reaction kits such as the OptiGene Genie III and their lyophilised master mixes offers several advantages. The Genie III is easily portable, weather-resistant and runs on a rechargeable battery, making it ideal for use in the field. It measures amplification by fluorescence and has inbuilt software to interpret the results. This is advantageous for several reasons: it avoids subjective user interpretation of results that can occur with visual LAMP detection methods such as turbidity and colour change; and obviates the need to open reaction tubes post-reaction as necessary with lateral flow devices (LFD), thereby removing the risk of cross-contamination of other samples and equipment with amplified product. It is also possible to obtain commercially available kits with lyophilised reagents and LAMP primers together in tubes ready for resuspension and use, to further streamline assay set up.

Dukes et al. (2006) established that the RT-LAMP primer set was able to amplify all serotypes of FMDV and demonstrated its exclusivity as other viruses that cause similar pathology or are genetically related to FMDV were not amplified. Howson et al. (2017) established that the same primers in a lyophilised OptiGene kit very similar to that used in this publication amplified isolates of serotypes O, A, SAT1, SAT2 and Asia 1, and did not amplify two other vesicular disease viruses. Using the same primer set and a similar lyophilised OptiGene kit we would expect the assay to have a similar specificity. We have successfully tested three serotypes (O, A and Asia 1) by amplifying a panel of RNA extracted FMDV samples that were available to us and circulating in our part of the world. Diseases in Australia, where the assay is being rolled out for use, with similar clinical symptoms to FMD (Ovine Footrot, Bovine Parapox and UAUS) tested negative in the RT-LAMP assay. This indicates no cross reactivity and a DSp approaching 100% for the FMDV RT-LAMP assay in both sheep and cattle.

The analytical sensitivity (LOD) based on the RNA standard was estimated to be around 1,000 copies, when diluted in EB buffer or VTM, which is the recommended matrix for collected swab samples to be placed in. Collection of swabs in PBS though may lead to a tenfold drop in sensitivity. Similar results were seen for the FMDV serotype O specific RT-LAMP assay, which included swarm primers for increased specificity, and also had a detection limit of 1,000 RNA copies (Lim et al., 2018). However, sensitivity was greater in the rRT-PCR, which was able to detect down to 10 copies, and in the original FMD RT-LAMP assay that was reported to detect 10 copies in the original publication (Dukes et al., 2006), and 10 copies/µl in a subsequent study (Howson, Armson, et al., 2017). The same primers and reaction temperatures, and similar reagents were also used in the Howson study

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(commercial master-mix, ISO-001, OptiGene Ltd.), but the difference in sensitivity may be due to the different polymerase in the kit (ISO-004) used in this study which is designed for faster amplification.

Despite the reduced analytical sensitivity, our FMDV LAMP assay was shown to work well in the field using unextracted samples. Dukes and colleagues (2006) were able to detect 83% (81/98) of field virus samples as positive in their RT-LAMP assay, using extracted RNA. In this study, using unextracted field samples and less sample material, we obtained comparable results. Following the OIE recommended BLCM approach when insufficient reference samples are available and a Frequentist approach (assuming sufficient reference samples) we arrived at similar estimates for DSe and DSp (79% and >99%, respectively) on unextracted samples (Bhutan and Australia). This is likely due to the superior accuracy of the reference test (rRT-PCR), and to the similar biological principles of both tests being compared (Comin et al., 2013). An advantage of the BLCM is that it facilitates the estimation of Se, Sp and prevalence across all tests and populations, i.e. two tests and two populations used in this study and makes no assumption regarding the true disease status of samples. For the rRT-PCR, the estimates for Se and Sp were very similar to published results (Goris et al., 2009).

Because of the similar biological principles of LAMP and rRT-PCR (detection of nucleic acid sequences that code for non-structural proteins) a conditional dependence model was chosen for data analysis. Although results for all samples tested are shown in the tables, only results from cattle were used to determine diagnostic Se and Sp and prevalence.

During field work it was observed that those samples that returned a negative result for FMD by the RT-LAMP assay had a rRT-PCR C_T value of >30. This may be a result of the lowered sensitivity of this assay compared to earlier studies, but is also likely attributable to less virus sample being added to the reactions – 1µl of unextracted sample in RT-LAMP versus 5µl of extracted sample in rRT-PCR – and the highly evolving nature of RNA viruses and potential sequence mismatches in primer binding sites (King et al., 2006).

Although LAMP enzymes are known to be tolerant to sample inhibitors, using unextracted samples increases the chances of false negatives occurring. Reducing the volume of sample used means less inhibitors are introduced into the reaction - and enabled the use of user-friendly 1µl disposable inoculation loops - but the addition of an IPC was designed to further counter the problem. Multiplexing in LAMP can be difficult as additional targets and primers in the reaction can lead to interaction between primers sets and result in non-specific amplification. A competitive IPC, however, is a different target that is amplified by the same primer set. The competitive IPC was successfully amplified, and the resulting T_a produced was approximately 1.5°C lower than that of the RNA standard or FMDV, making its amplification easily distinguishable. Its use in the field allowed negative LAMP samples to be confirmed as FMDV negative by ruling out inhibitors. Though less than 15% of field

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samples in Bhutan, and less than 12% of Australian differential disease samples needed to be diluted to overcome inhibition before a final verdict could be determined.

The purpose of this study was to further develop and validate a "fit for purpose" in-field RT-LAMP assay for FMD that could be used by non-laboratory trained staff. We did this by adapting a primer set that had successfully been used in the field with lyophilised reagents and unextracted samples and streamlined the set up by using a fast lyophilised commercial kit, removing dilution steps, using inoculation loops and introduced an IPC to identify possible inhibition. The intended end user of this assay is the state government field veterinarians in Victoria, Australia, to assist disease surveillance and exclusion activities and complement current diagnostic capability when investigating suspect cases of FMD. Robustness and ruggedness of the LAMP assay was challenged under field conditions in Bhutan and on almost 200 samples taken in Victoria with satisfactory results, and the use of an IPC provided constant quality assurance of results. Repeatability of the assay in different locations by different operators was good, and although CV% was higher with smaller volumes of the ssRNA control and non-laboratory trained staff, the assay continued to achieve satisfactory results.

Under current policy, confirmatory diagnosis by the OIE-approved "reference standard" rRT-PCR will continue to be conducted on all FMD-suspect investigations, including negative ones. It is important to clarify and highlight that all samples that are tested by RT-LAMP as a primary line of diagnosis in the field will be followed-up using the established rRT-PCR and results interpreted by trained laboratory personnel and in accredited laboratories. LAMP provides preliminary diagnostic information to support early detection and decision-making for government emergency animal disease responders and mitigate potential impacts for industry.

The LAMP-FMDV test is a simple, in-field diagnostic tool that has the potential for rapid and reliable detection of FMDV. Through its use there is the potential for improvements in the ability to detect disease earlier, mitigate sample transportation delays and reduce the size and spread of an outbreak through early implementation of control measures. This technology has the potential to support jurisdictions to protect the health, welfare and trade of their valuable livestock industries.

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Conflict of Interest Statement

The authors have no conflict of interest.

Ethics Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as no clinical trials took place. All samples were collected from animals by veterinarians as part of their routine diagnostic practices.

Data Availability Statement

Research data was not required to be shared at the time of submission.

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Table 1. FMD viral RNA obtained from the FMD Regional Reference Laboratory (RRL) in Thailand determined inclusivity and comparative sensitivity between rRT-PCR and RT-LAMP.

Sample Name	RT-LAMP			rRT-PCR	Serotype/Topotype/ Lineage
	T_A (°C)†	T_P (min:sec)‡	Result	C_T§	
179/16	88.6	7:15	+	19.73	O/ME-SA/Ind2001e
22/17	88.8	6:57	+	24.88	O/ME-SA/Ind2001e
225/16	88.8	8:42	+	30.00	O/ME-SA/Ind2001e
25-1/17	88.8	7:30	+	19.23	O/ME-SA/Ind2001e
44/17	88.8	7:00	+	23.87	O/ME-SA/Ind2001e
443/16	88.9	4:57	+	18.17	O/ME-SA/Ind2001e
445-2/16	88.9	4:57	+	19.74	O/ME-SA/Ind2001e
45/17	88.8	5:15	+	19.47	O/ME-SA/Ind2001e
47/17	88.8	4:42	+	25.92	O/ME-SA/Ind2001e
49/17	88.9	5:42	+	26.93	O/ME-SA/Ind2001e
surat-1/17	88.8	6:27	+	22.45	O/ME-SA/Ind2001e
166-2/16	88.9	4:00	+	16.17	O/SEA/Mya-98
169-2/16	88.9	4:45	+	14.47	O/SEA/Mya-98
174-1/16	89.0	4:15	+	17.61	O/SEA/Mya-98
180/16	89.0	4:15	+	16.19	O/SEA/Mya-98
181/16	89.0	4:30	+	21.75	O/SEA/Mya-98
182-1/16	89.0	4:45	+	18.72	O/SEA/Mya-98
184/16	89.0	4:00	+	15.89	O/SEA/Mya-98
190/16	89.0	5:15	+	26.71	O/SEA/Mya-98
192/16	89.0	5:15	+	24.04	O/SEA/Mya-98
194-5/16	89.0	4:45	+	20.32	O/SEA/Mya-98
196-3/16	89.0	3:45	+	16.78	O/SEA/Mya-98
206/16	89.0	4:30	+	17.84	O/SEA/Mya-98
208/16	89.0	4:45	+	18.19	O/SEA/Mya-98
213/16	89.0	4:30	+	20.18	O/SEA/Mya-98
216-2/16	89.0	3:45	+	18.77	O/SEA/Mya-98
220/16	89.0	4:15	+	15.52	O/SEA/Mya-98
221-4/16	89.0	3:45	+	17.32	O/SEA/Mya-98
227-2/16	89.0	4:12	+	14.68	O/SEA/Mya-98
3-2/17	89.0	6:42	+	30.00	O/SEA/Mya-98
354-3/16	89.0	4:42	+	31.37	O/SEA/Mya-98
360/16	89.0	4:27	+	18.21	O/SEA/Mya-98
394-2/16	89.1	4:42	+	20.57	O/SEA/Mya-98
396-1/16	89.1	3:57	+	17.81	O/SEA/Mya-98
418-1/16	89.1	4:42	+	21.28	O/SEA/Mya-98
421-2/16	89.1	4:42	+	23.77	O/SEA/Mya-98
10/17	89.1	4:57	+	23.18	A/ASIA/Sea-97
12/17	89.1	4:27	+	18.89	A/ASIA/Sea-97
15/17	89.1	7:27	+	19.68	A/ASIA/Sea-97
19/17	89.2	5:42	+	19.61	A/ASIA/Sea-97
2/17	89.0	5:42	+	19.35	A/ASIA/Sea-97
24/17	89.2	6:00	+	20.78	A/ASIA/Sea-97
258-2/16	88.7	7:27	+	30.00	A/ASIA/Sea-97
30/17	89.2	8:15	+	21.66	A/ASIA/Sea-97
31/17	89.3	4:00	+	18.78	A/ASIA/Sea-97
377/16	89.3	6:57	+	25.54	A/ASIA/Sea-97
383/16	89.3	5:12	+	23.98	A/ASIA/Sea-97

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447/16	89.4	4:42	+	18.52	A/ASIA/Sea-97
46/17	89.3	5:42	+	23.96	A/ASIA/Sea-97
6/17	89.0	4:12	+	16.63	A/ASIA/Sea-97
MYA-26/17	88.8	5:42	+	27.43	Asia 1/ASIA/G-VIII

† T_A – annealing derivative temperature

‡ T_P – time to positive, in minutes: seconds

§ C_T value (threshold cycle) from rRT-PCR, performed by RRL

Table 2. Comparative diagnostic sensitivity of FMDV RT-LAMP assay and the rRT-PCR assay using clinical samples collected in Bhutan.

Animal number	Species	Sample type	Lesion age (days)	RT-LAMP				rRT-PCR
				T_P (min:sec)	T_A (°C)	Result	Sample dilution	C_T value
F1	Bovine	mouth	8	19:00	89.3	+		36.02
F2	Bovine	mouth	2	9:30	88.9	+		28.49
F3	Bovine	mouth, fibrin	4	13:45	88.8	+		24.56
F4	Bovine	mouth	4			-		30.41
F5	Bovine	mouth	4	17:45	88.8	+		27.81
F6	Bovine	mouth	3			-		34.10
F7	Bovine	mouth	2-3	13:00	88.7	+		35.17
F8	Bovine	mouth	np	14:27	88.9	+		31.22
F9	Bovine	mouth	5	17:00	88.3	+		30.48
F10	Bovine	mouth	np	17:27	88.9	+		35.04
F1	Bovine	foot		16:15	88.9	+		24.26
F2	Bovine	foot		17:15	88.9	+		20.22
F5	Bovine	foot		12:00	88.9	+		22.02
A1	Bovine	mouth		11:15	88.4	+		26.07
A2	Bovine	mouth				-		34.01
A3	Caprine	mouth				-	1/50†	33.90

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A4	Bovine	mouth	14:12	88.5	+	1/50	27.37
A5	Bovine	mouth	17:45	88.0	+		28.40
A6	Bovine	mouth			-		30.80
A7	Bovine	mouth	12:42	88.8	+		14.40
A8	Bovine	mouth	8:45	88.4	+		19.80
A9	Bovine	mouth	8:57	88.6	+		18.20
A10	Bovine	mouth	11:00	88.9	+	1/50	23.26
A11	Bovine	mouth	7:42	88.6	+		15.70
A12	Bovine	mouth	6:00	89.0	+	1/50	18.24
A13	Bovine	mouth	9:42	88.7	+	1/50[†]	18.18
A14	Bovine	mouth			-		32.30
A15	Bovine	mouth	10:27	88.6	+	1/50[†]	20.70
A16	Bovine	mouth	16:12	88.2	+		16.60
A17	Bovine	mouth			-	1/50[†]	30.40
A18	Bovine	mouth	14:12	88.4	+		20.90
A19	Bovine	mouth	9:42	89.0	+	1/50[†]	18.00
A20	Bovine	mouth	7:45	88.6	+	1/50	13.50
A21	Bovine	mouth	11:57	88.1	+		17.30

Samples have been designated as field (F) samples freshly collected from an outbreak or archival (A) samples that have been previously collected in Bhutan. Unextracted samples were used directly in the RT-LAMP assay, with most of the freshly collected samples being run in the field. Time to positive (T_P) and the anneal derivative temperature (T_a) are recorded. Some archival samples needed to be diluted in Buffer EB before a final RT-LAMP result could be obtained, either due to inhibition[†] as indicated by the internal positive control, or a T_a outside the expected range of 87.9°C to 89.9°C. All samples were extracted and evaluated with the rRT-PCR assay with the C_T values listed. np = none present.

Table 3. Relative diagnostic sensitivity (DSe) and specificity (DSp) point estimates and upper and lower limits for 95% confidence intervals (95% CI) for LAMP in relation to rRT-PCR assay.

Sample origin and type	Parameter	# of positive or negative	# of samples tested	Point estimate	95% CI

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FMD infected animals:					
Bhutan (oral and foot swabs)†	DSe	27	33	0.82	0.65–0.93
Thailand (RNA extracts)	DSe	51	51	1.00	0.93–1.00
FMD free animals:					
Australia, Victoria, cattle‡	DSp	96	96	1.00	0.96–1.00
Australia, Victoria, sheep §	DSp	90	90	1.00	0.96–1.00

† One sample that consisted of a goat was not included in the analysis (tested negative).

‡ Field samples from symptomatic cattle infected with Bovine Papular Stomatitis (Parapox) (n = 11), Upper Alimentary Ulcerative Syndrome (UAUS) (n = 85) and

§ ovine footrot.

Table 4. Detection of the analytical sensitivity of the FMDV RT-LAMP assay, compared to the Tetracore rRT-PCR.

RNA Copy #	RT-LAMP		rRT-PCR	
	Results (# positive/total)	T_P (Mean ± SD)	Results (# positive/total)	C_T (Mean ± SD)
1×10^6	3/3	5.11 ± 0.17	3/3	19.95 ± 0.07
1×10^5	3/3	5.69 ± 0.05	3/3	23.16 ± 0.04
1×10^4	3/3	7.03 ± 0.55	3/3	26.87 ± 0.13
1×10^3	3/3	9.69 ± 1.68	3/3	30.31 ± 0.13
1×10^2	2/3	13.71 ± 4.46	3/3	34.29 ± 0.93
1×10^1	0/3		3/3	36.65 ± 2.46
1×10^0	0/3		2/3	37.53 ± 2.05

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Table 5. Comparative diagnostic specificity of FMDV RT-LAMP assay and the rRT-PCR assay using unextracted clinical samples from sheep and cattle collected in Australia.

	RT-LAMP positive	RT-LAMP negative			
		No dilution	1/10 dilution	1/100 dilution	1/1000 dilution
rRT-PCR positive	0	0	0	0	0
rRT-PCR negative	0	164	14	7	1
Total	0	186			

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Table 6. Repeatability analysis of FMD RT-LAMP assay as seen by the performance of the positive controls.

Location	Control	Variability of T_P value (minutes)				Variability of T_A value (°C)			
		Mean	SD	CV (%)	95% CI	Mean	SD	CV (%)	95% CI
Thailand	IPC (n = 9)	6.94	0.64	9.20	7.15-6.72	87.46	0.11	0.12	87.50-87.42
	Standard† (n = 19)	4.85	0.18	3.75	4.89-4.81	89.03	0.08	0.09	89.05-89.01
Bhutan	Standard† (n = 23)	9.28	1.11	11.93	9.51-9.05	88.75	0.10	0.11	88.77-88.73
Australia	Standard† (n = 77)	8.40	2.20	26.19	8.65-8.15	88.95	0.11	0.12	88.96-88.94

The FMD RT-LAMP assays have been predominantly run by a different operator in each country using the RNA standard† primarily as the positive. The mean and standard deviation (SD) of the time to positive (T_P) and annealing derivative temperature (T_A) of the positive controls used in these assays are reported along with the resulting Coefficient Variation (CV) and 95% confidence intervals (CI).

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Table 7. Sensitivity and specificity of LAMP and Tetracore rRT-PCR assay and prevalence estimates for cattle samples from Bhutan and Australia using Bayesian Latent Class Model.

	Node	Median	95% HPD
Tetracore	Sensitivity	0.958	(0.915, 0.983)
	Specificity	0.985	(0.961, 0.996)
LAMP	Sensitivity	0.792	(0.645, 0.899)
	Specificity	0.990	(0.957, 1.000)
Prevalence	Diseased (Bhutan)	0.980	(0.895, 0.999)
	Non-diseased (Australia)	0.003	(0.000, 0.034)
ρ	Diseased	0.146	(-0.101, 0.456)
	Non-diseased	0.358	(0.009, 0.879)

ρ represents the conditional correlation between the two test outcomes in truly diseased and non-diseased animals respectively.

95% HPD = highest probability density region.

Deviance information criterion (DIC) = 23.7 and approximate true number of parameters (pD) = 8.4.

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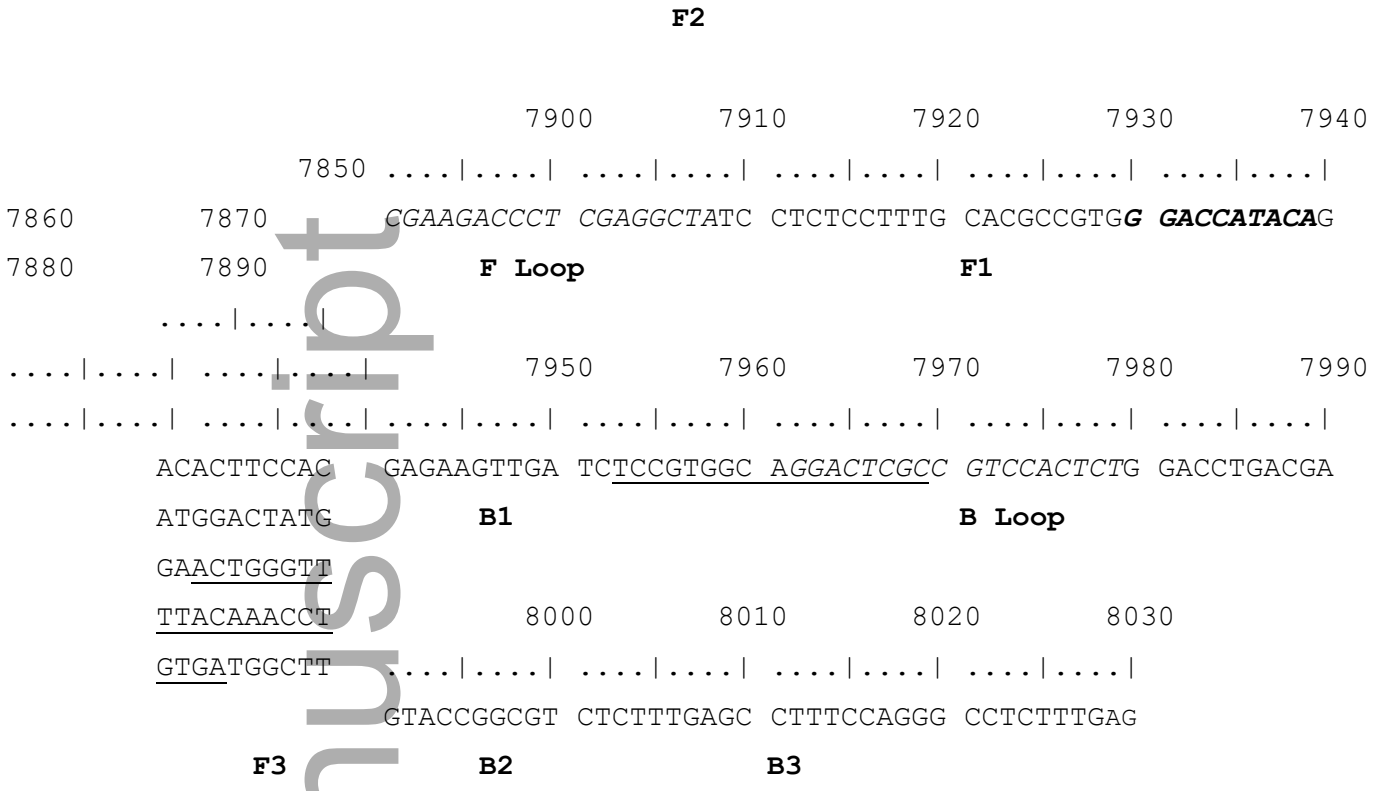

Figure 1. Position of the RT-LAMP primer sites in the 3D gene target of FMDV type O strain UKG/35/2001. The external primers F3 and B3 are shown in dark grey, the binding locations of the internal primers FIP and BIP are shown in light grey and the loop primers are in italics. Between the forward and backward LAMP primer sites is a ten-nucleotide region of sequence, shown in bold italics, which was altered to create the IPC that would amplify with the same primers. The forward and reverse primer sites of the Tetracore rRT-PCR are underlined. Genome position according to GenBank Accession AJ539141.

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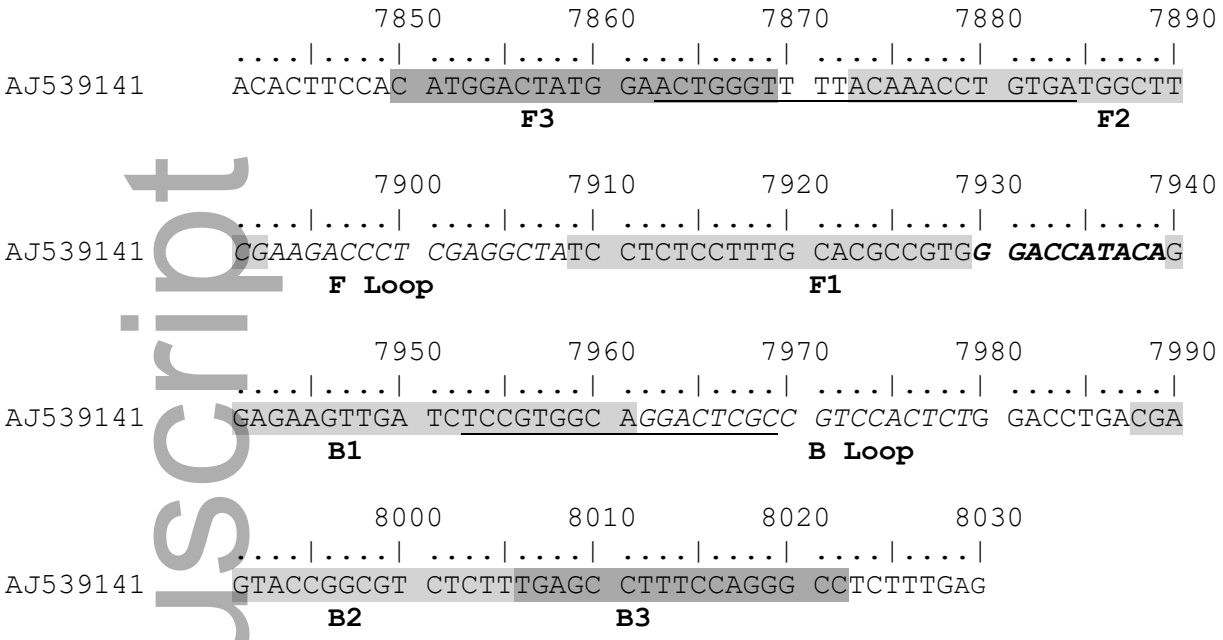

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