

**Novel reverse transcription loop-mediated
isothermal amplification for rapid detection
of foot-and-mouth disease virus**

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Summary. Speed is paramount in the diagnosis of foot-and-mouth disease (FMD) and simplicity is required if a test is to be deployed in the field. The development of a one-step, reverse transcription loop-mediated amplification (RT-LAMP) assay enables FMD virus (FMDV) to be detected in under an hour in a single tube without thermal cycling. A fragment of the 3D RNA polymerase gene of the virus is amplified at 65 °C in the presence of a primer mixture and both reverse transcriptase and *Bst* DNA polymerase. Compared with real-time RT-PCR, RT-LAMP was consistently faster, and ten copies of FMDV transcript were detected in twenty-two minutes. Amplification products were detected by visual inspection, agarose gel electrophoresis, or in real-time by the addition of a fluorescent dye. The specificity of the reaction was demonstrated by the absence of amplification of RNA from other viruses that cause vesicular diseases and from that of genetically related picornaviruses. Diagnostic sensitivity was validated by the amplification of reference FMDV strains and archival material from field cases of FMD. In comparison with the performance of the established diagnostic TaqMan[®] assay, RT-LAMP appears to be sensitive, rapid, specific, and cost-effective, with the potential for field deployment and use by developing countries for FMDV surveillance.

Introduction

The 2001 outbreak of foot-and-mouth disease (FMD) in the United Kingdom highlighted the devastating potential of this disease to decimate the livestock industry and economy of a country previously free from the disease. This epizootic lasted for 7 months, requiring the slaughter of more than 6 million animals for

its control, and is estimated to have cost £8 billion to the national economy [2]. Once the UK government introduced the control strategy of slaughtering the animals on infected premises within 24 h and those on dangerous contact and contiguous premises within 48 h, confirmation of clinical signs by laboratory diagnosis was no longer achievable. Therefore, the development of technologies that provide rapid and sensitive diagnosis of FMD that ideally can be deployed *in situ* without transferring the samples to a central laboratory is a current research priority.

FMD is an extremely contagious viral disease affecting domesticated ruminants and pigs, in addition to over 70 species of wildlife [1, 4]. FMD virus (FMDV), a positive-stranded RNA virus, is the type member of the genus *Aphthovirus* within the family *Picornaviridae*. There are 7 recognised serotypes of FMDV denoted O, A, C, SAT1, SAT2, SAT3, and Asia 1), which can be further sub-divided into distinct topotypes based on phylogenetic analysis [13].

Established diagnostic techniques (antigen-trapping ELISA and virus isolation) require the presence of intact viral antigens and/or live virus, which depends on sample quality. However, molecular tools, such as reverse transcription PCR (RT-PCR) detect viral genomes (or fragments thereof), and can be used on samples which fail to grow in tissue culture, enabling detection of partially degraded and previously infectious substances. RT-PCR has been shown to be a powerful and sensitive tool in FMDV detection [19], and since the 2001 UK outbreak it has been improved and automated so that its performance now exceeds that of the conventional methods for routine diagnosis in terms of both speed and sensitivity [21]. The recent development of portable equipment for PCR has made molecular diagnosis of FMD possible in the field [3, 6, 9]. However, this approach relies on precision thermocycling, requiring instrumentation which can be fragile, prohibitively expensive, and that will require decontamination when transferred from one site to another.

Loop-mediated amplification (LAMP) also amplifies specific nucleotide sequences [17]. However, unlike PCR, a denatured template is not required [15] and DNA is generated in abundance so that positive LAMP reactions can be visualised with the naked eye [11, 14 (Fig. 1)]. LAMP has been reported to have equivalent (or improved) analytical sensitivity when compared to PCR methods for virus detection [18]. This report describes the development of a one step, single-tube, accelerated, FMDV-specific RT-LAMP assay for the rapid detection of all seven serotypes of the virus.

Materials and methods

Virus isolates

Virus isolates were either in the form of suspensions made from epithelia collected from vesicular lesions or virus stocks derived from their passage in cell culture. The original samples had been submitted to and stored at the FAO World Reference Laboratory for FMD (WRL) at Pirbright. Viruses known to be genetically related to FMDV or to cause similar clinical signs were examined to evaluate the FMDV-specificity of the assay under development. These viruses included swine vesicular disease virus (SVDV), vesicular exanthema of swine

Table 1. Details of oligonucleotide primers used for RT-LAMP amplification of the 3D polymerase gene of FMD virus

Primer name	Type	Length	Genome position*	Sequence (5'–3')
F3	Forward Outer	20-mer	7850–7869	CATGGACTATGGAACTGGGT
B3	Reverse Outer	17-mer	8006–8022	GGCCCTGGAAAGGCTCA
FIP	Forward Inner (F1C + TTTT + F2)	45-mer; F1C, 21mer; F2, 20mer	F1C, 7929–7909; F2, 7873–7892	CACGGCGTGCAAAGGAGAGGA TTTTACAAACCTGTGATGGCTTCG
BIP	Reverse Inner (B1C + TTTT + B2)	44-mer; B1C, 22-mer; B2, 18-mer	B1C, 7961–7940; B2 – 7988–8005	GGAGAAGTTGATCTCCGTGGCA TTTTAAGAGACGCCGGTACTCG
F Loop	Forward Loop	18-mer	7891–7908	TAG CCT CGA GGG TCT TCG
B Loop	Reverse Loop	18-mer	7962–7979	GGA CTC GCC GTC CAC TCT

*Genome position according to the FMD virus strain O UKG 35/2001 (Genbank Accession number AJ539141)

virus (VESV), San Miguel sea lion virus (SMSV), bovine calicivirus, equine rhinitis A virus (ERVA), and bovine rhinoviruses (BRV).

RNA extraction

Total RNA was extracted from 200 µl original epithelial suspensions or from cell culture supernatants using RNeasy Mini-Kit (Qiagen). After elution in 50 µl nuclease-free H₂O, RNA samples were stored in aliquots of 10 µl at –80 °C until required.

RT-LAMP

The 3D RNA polymerase-encoding region of the FMDV genome was chosen as a suitable target, as it provides a relatively conserved amplicon of ~200 bp. Primers (given in Table 1), were designed using Primer Explorer V3 software (<https://biodb.netlaboratory.com/lamp/index.html>) against the sequence of the type O FMDV isolate UKG 35/2001 (Genbank Accession number AJ539141). The two outer primers (F3 and B3) help to displace the primary strand (Fig. 1). The inner primers (FIP and BIP, see Fig. 1 and Table 1) each have two distinct sequences corresponding to the sense and antisense sequence of the target. FIP comprises F1C (complementary to F1: see Fig. 2), a TTTT spacer and the F2 sequence. BIP comprises the B1C sequence (complementary to B1) a TTTT spacer, and the B2 sequence. Loop primers were designed manually to correspond to the regions between F1 and F2, and B1 and B2. Initial experiments were performed to optimise the assay by varying betaine, MgSO₄, and primer concentrations and amplification temperature in order to amplify cell culture virus isolates belonging to at least one member of each toptotype [13] from all seven serotypes (A: n = 6; O: n = 11; C: n = 7; Asia1: n = 4; SAT1: n = 3; SAT2: n = 9; SAT3: n = 4). Final reaction mixtures (25 µl volume) contained primers at a ratio of 5 pmoles external primers: 50 pmoles internal primers: 25 pmoles loop primers with 6 mM MgSO₄ (New England Biolabs) 1 M Betaine (Sigma), 16 U of *Bst* DNA polymerase (large fragment; New England Biolabs), 1X ThermoPol buffer (New England Biolabs) 3 U ThermoScript™ RT (Invitrogen), with 2 µl total RNA as template.

Interpretation of RT-LAMP results

RT-LAMP results were visualised by both agarose gel analysis and by adding 5 µl of the intercalating dye Picogreen® (Molecular Probes). To confirm that amplification products

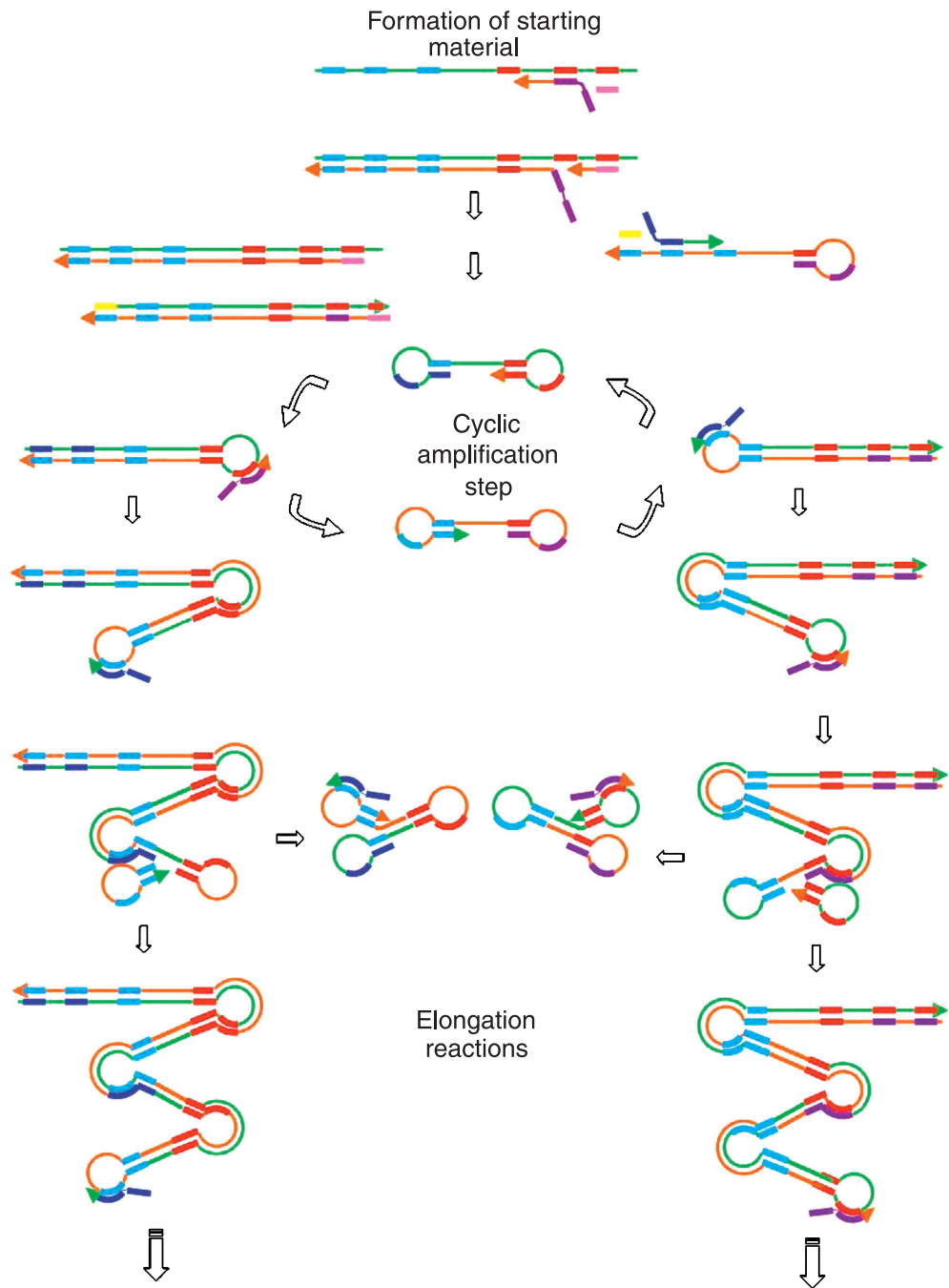


Fig. 1. Loop Mediated Amplification (adapted from [16]). Six regions of nucleotide sequence (F1, F2, F3: coloured light blue; B1, B2 and B3 coloured red) are used to design two sets of primers. Inner primers (FIP and BIP: dark blue and purple) contain F2 and B2 sequences attached to the complement of F1 and B1. Outer primers (F3 and B3: yellow and pink) displace the FIP and BIP-amplified strands, which then form a hairpin to create the starting loop for cyclic amplification. Amplification then proceeds in a cyclical manner, each previous strand being displaced during elongation, with the addition of extra loops with each round, eventually producing multiple products of predictable sizes. An additional set of primers (Floop and Bloop [not shown]) specific to the loops formed between F1 and F2 and B1 and B2 respectively, accelerates the reaction [16]

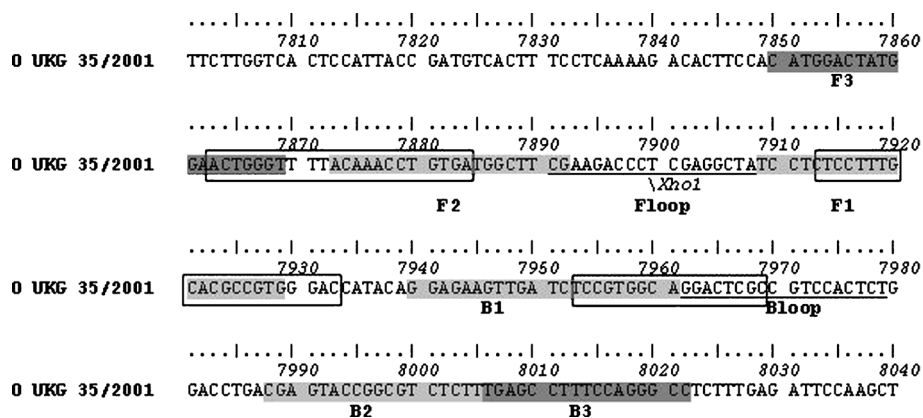


Fig. 2. Position of oligonucleotide primers used for RT-LAMP amplification of the 3D polymerase gene of FMDV showing *Xho* I site used for restriction analysis, and location of TaqMan[®] primers and probe. Positions of external RT-LAMP primers are shown in dark grey, internal RT-LAMP primer positions are shown in light grey and the position of the TaqMan[®] primers and probe are indicated by the boxes. Genome position according to the FMDV strain O UKG 35/2001 (Genbank Accession number AJ539141)

consisted of the stem loop structures predicted, 2 μ l of the RT-LAMP reaction from type O UKG 35/2001 was digested with *Xho* I at 37 °C for one hour and the products visualised by agarose gel analysis.

Construction of RNA standard to determine analytical sensitivity of RT-LAMP assay

The analytical sensitivity of the RT-LAMP reaction was determined using a standard generated from a T7 RNA polymerase transcript from an O UKG 35/2001 3D amplicon. The amplicon was generated by including the T7 promoter sequence (5'-TAA TAC GAC TCA CTA TAG GGA G-3') at the 5' end of the forward external primer, F3, and amplifying from template cDNA (isolate O UKG 35/2001) using B3 reverse primer. Reaction conditions (25 μ l volume) consisted of 10 pmoles of each primer and 1X BioMix ready-to-go reagent (Bioline) and the following cycling times and temperatures: 94 °C for 30 s, and 35 cycles of 94 °C for 10 s, 55 °C for 15 s, and 72 °C for 10 s. After agarose gel analysis, the PCR product was used as template for *in vitro* transcription (MEGAscript[™] T7 kit, Ambion) and the resultant transcript treated with DNase I. The RNA product consisting of the 175 nucleotide LAMP target, was quantified using the Agilent 2100 Bioanalyzer; transcript copy number was then calculated and standards were prepared by 10-fold serial dilution of template.

Comparison of speed and sensitivity with TaqMan[®] real-time RT-PCR

The current “gold standard” for molecular diagnosis of FMD is TaqMan[®] real-time RT-PCR [3, 19–21]. An established real-time RT-PCR specific for the 3D RNA polymerase gene [3] was compared to RT-LAMP, as the target regions of the two assays overlap, enabling the same set of RNA standards to be used (Fig. 2). The cDNA for TaqMan[®] amplification was synthesised using 6 μ l total RNA in a 15 μ l reaction using TaqMan[®] Reverse Transcription Reagents (Applied Biosystems, Roche). Real-time PCR was carried out in an MX4000 thermocycler (Stratagene) using TaqMan[®] reagents (Applied Biosystems) according to the manufacturer’s protocol. In each 25 μ l PCR reaction, 22.5 pmoles of each primer and 12.5 pmoles of probe were used to amplify 2.5 μ l cDNA.

In order to monitor the RT-LAMP reaction in real time, the reaction was transferred to the Stratagene MX4000 real-time thermocycler. The intercalating dye Picogreen[®] (Molecular Probes) was added to the RT-LAMP reaction at a final dilution of 1:1000. To control for thermal effects on fluorescent emission, 6-ROX (Molecular Probes) was added as a reference dye at a final concentration of 100 ng/ml. The same reaction conditions were used for the end-point reaction (described above), except that the MgSO₄ concentration was lowered to 4 mM. Raw fluorescence data derived from Picogreen[®] (equivalent to SYBR[®] green) was collected in real time during the amplification. An increase in fluorescence indicates the amplification of DNA, but the use of 6-ROX as a passive reference dye enables any change in fluorescence due to the temperature of incubation alone to be removed from the amplification signal. The T_p value was designated as the time at which the fluorescence of the RT-LAMP reaction rose above a set threshold. The threshold value for positivity by the real-time RT-LAMP assay was positioned above the background fluorescence of 'no template control' reactions as recommended for real-time PCR by PE Applied Biosystems.

Evaluation of performance on field samples

To determine the assay's performance on a variety of virus isolates from the field, samples of epithelia (n = 98) submitted to the FAO WRL for FMD were tested using both the diagnostic TaqMan[®] assay [3] and RT-LAMP. These epithelia were collected from suspect FMDV cases worldwide representing a broad coverage of the inter- and intra-typic genetic variation of FMDV.

Results

The RT-LAMP assay was initially optimised using viral RNA from the FMDV isolate O UKG 35/2001. The assay successfully amplified the 174-bp target sequence of the 3D polymerase gene at 65 °C in 60 min, as revealed by agarose gel

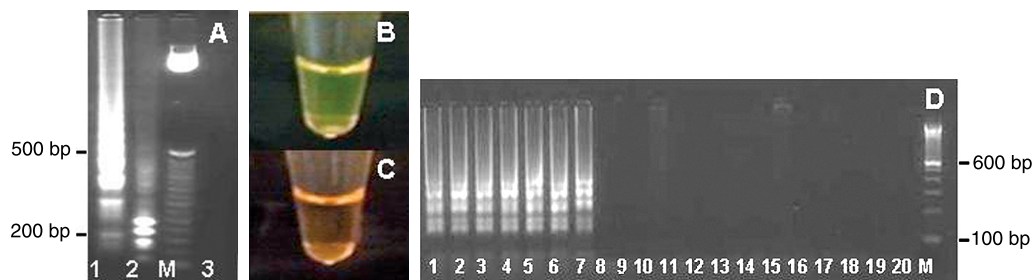


Fig. 3. **A:** Agarose gel electrophoresis and restriction analysis of the RT-LAMP product of the 3D gene of FMDV. Lanes: 1, RT-LAMP products of virus strain O UKG 35/2001; 2, RT-LAMP products digested with *Xho* I (131 bp and multiples thereof); 3, No template control; M, 25-bp molecular weight marker (Invitrogen). **B:** positive RT-LAMP reaction visualised in the reaction tube by the addition of Picogreen[®]. **C:** Negative RT-LAMP reaction in the reaction tube with Picogreen[®]. **D:** Amplification from all 7 serotypes of FMDV, but not from related viruses: Lanes: 1–7 = FMDV: O JAV 5/32, A₂₂ IRQ 24/64, C PHI 1/88, Asia 1 LEB 3/83, SAT1 BOT 1/68, SAT2 MAI 7/91, SAT3 BEC 1/65; 8–12 = SVDV: HKN 1/76, ITL 1/92, ITL 18/92, ITL 29/92, AUT 1/73; 13, 14 = San Miguel sea lion virus SMSV-1 and SMSV-9; 15 = bovine calicivirus; 16, 17 = VESV A48 and VESV H54; 18 = ERVA; 19 = BRV-1; 20 = BRV-2

electrophoresis. The reaction products, observed as a ladder-like pattern on the gel (Fig. 3A), were due to the formation of stem-loop DNAs of varying stem length and cauliflower-like structures with multiple loops formed by sequentially inverted repeats of the target sequence. This structure was confirmed by restriction analysis; products of predictable sizes comprising repeats of the same 131-bp motif (Fig. 2) were resolved on a gel (Fig. 3A). A green colour, produced by the intercalating dye Picogreen[®], corresponded exactly with the generation of products with the same ladder-like appearance (Fig. 3B). A negative reaction containing no template RNA (Fig. 3C) or RNA from a non-FMD virus (data not shown), as revealed by electrophoresis, corresponded to an orange colour in the presence of Picogreen[®] (Fig. 3C).

The specificity of the reaction for FMDV was determined by checking the cross-reactivity of the assay with isolates of swine vesicular disease virus (SVDV: $n = 5$), vesicular exanthema of swine virus (VESV: $n = 2$), San Miguel sea lion virus (SMSV: $n = 2$), bovine calicivirus ($n = 1$), and both bovine rhinovirus and equine rhinitis A virus (BRV: $n = 2$ and ERVA; $n = 1$) – viruses that cause similar pathology to or are genetically related to FMDV. The primer set demonstrated a high degree of specificity for FMDV by amplifying members of all seven

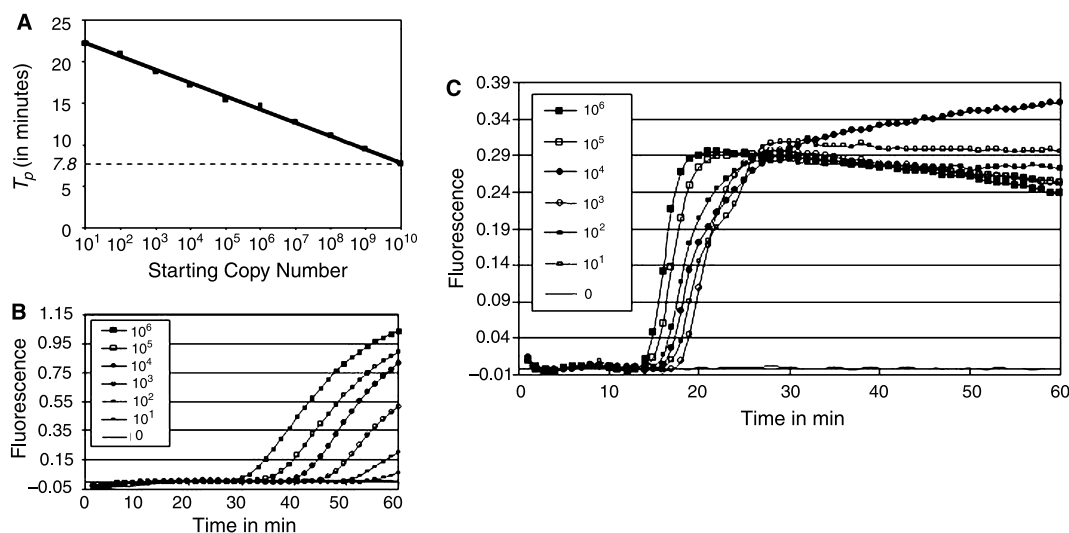


Fig. 4. Comparative speed and sensitivity of real-time RT-LAMP and TaqMan[®] RT-PCR for the detection of FMD virus. **A:** Quantitative determination of virus concentration. Standard curve generated for RT-LAMP FMD assay by plotting T_p (in minutes) derived from RNA templates of known copy number. Dotted line indicates that 10^{10} copies were detected in 7.8 min. **B:** Sensitivity and speed of TaqMan[®] assay for FMDV is 10 copies of starting RNA, which are detected in 55 min (1 cycle of TaqMan[®] = 1.5 min). **C:** Sensitivity and speed of RT-LAMP assay for FMDV as monitored by real-time measurement of fluorescence emitted by Picogreen[®]. Shown from left to right are curves of decreasing copy number of RNA template from 10^6 to 10 copies. Sensitivity limit of the assay is 10 copies of RNA, which are detected in ~20 min. ■ 10^6 copies; □ 10^5 copies; ▣ 10^4 copies; □ 10^3 copies; ● 10^2 copies; ○ 10 copies of RNA template

serotypes of FMDV but yielded negative results for all the other viruses tested (Fig. 3D).

Monitoring the reaction in real time was made possible by transferring the RT-LAMP assay to a real-time thermocycler. The fluorescence emitted by Picogreen[®] was normalised by the inclusion of an internal reference dye and the real-time analysis revealed that the amplification of 10^{10} copies of starting RNA template could be detected in 7.8 min (Fig. 4A). Accumulation of product was indicated by a continuous increase in fluorescence, while there was no change in background fluorescence in negative control wells which did not contain template (Fig. 4C). A standard curve depicting a linear relationship between the concentration of starting template and T_p was derived by real-time monitoring of the amplification of different copy numbers of RNA ranging from 10^{10} to 10 copies (Fig. 4A).

Comparison of speed and sensitivity with TaqMan[®] real-time RT-PCR

To determine the detection limit of the assay, a synthetic transcript consisting of the target region from O UKG 35/2001 was constructed. From 10^6 copies of starting material, RT-LAMP product was detected in 15 min (Fig. 4A and C), whilst 10 copies were detected in 22.2 min. Converting TaqMan[®] cycle number to time (each cycle takes 90 seconds) allows the speed of the two assays to be compared. Although TaqMan[®] was able to amplify from the same amount of

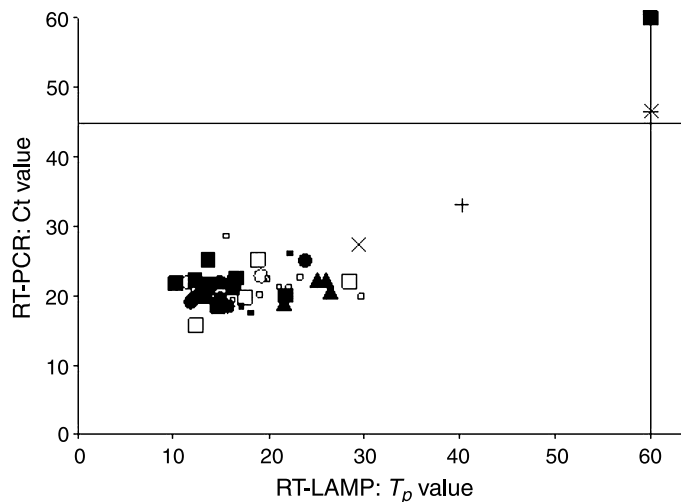


Fig. 5. Comparative specificity of real-time RT-LAMP and TaqMan[®] assays for FMDV using diverse cell-culture-grown virus strains. All isolates beneath the horizontal line ($Ct \leq 45$) and to the left of the vertical line ($T_p \leq 60$) are positive by RT-LAMP and TaqMan[®] assays. Those isolates above the horizontal line ($Ct \geq 45$) and to the right of the vertical line ($T_p \geq 60$) are deemed negative by both assays. Neither assay amplifies SVDV, and RT-LAMP detects as diverse a selection of FMDV isolates as TaqMan[®]. ○: serotype A; □: serotype O; ■: serotype C; ●: serotype Asia 1; □: serotype: SAT1; ◆: serotype SAT2; ▲: serotype SAT3 +: No virus detected; ×: SVD

Table 2. Comparative diagnostic sensitivity for TaqMan[®] RT-PCR and RT-LAMP assay systems for the detection of FMDV in field samples

FMDV Serotype	Strain	TaqMan (Ct)	Interpretation	RT-LAMP (T_p)	Interpretation
A	ALB 4/96	28.46	pos	55.28	pos
A	BHU 35/2003	32.72	pos	46.28	pos
A	BHU 41/2002	No Ct	neg	No T_p	neg ^c
A	BHU 6/2003	27.93	pos	17.045	pos
A	BHU 7/2003	35.32	pos	51.24	pos
A	BRA 2/95	26.39	pos	No T_p	neg ^a
A	IRN 10/2003	21.365	pos	22.045	pos
A	IRN 3/96	21.93	pos	29.33	pos
A	IRN 4/2000	32.18	pos	17.555	pos
A	IRN 5/2003	24.09	pos	16.865	pos
A	IRN 7/2000	25.11	pos	36.215	pos
A	IRN 7/2003	37.4	pos	25.835	pos
A	IRQ 59/2002	22.18	pos	21.11	pos
A	IRQ 99/2002	28.81	pos	54.57	pos
A	KEN 15/98	36.75	pos	53.73	pos
A	MAY 14/97	34.82	pos	22.63	pos
A	NEP 23/96	37.5	pos	No T_p	neg ^a
A	PAK 28/2002	16.23	pos	20.835	pos
A	PAK 9/2003	22.19	pos	56.59	pos
A	SYR 5/2002	26.08	pos	15.885	pos
A	TAI 4/2003	20.64	pos	21.235	pos
A	TUR 1/2000	22.95	pos	22.755	pos
A	TUR 14/2002	48.015	neg	56.165	pos ^b
A	TUR 15/2002	34.85	pos	36.975	pos
A	TUR 16/2002	40.515	pos	30.355	pos
A	TUR 4/2002	35.84	pos	35.51	pos
A	YEM 2/98	36.26	pos	24.42	pos
O	NGR 16/2001	No Ct	neg	No T_p	neg ^c
O	BHU 22/2003	26.27	pos	18.16	pos
O	BHU 48/2003	26.17	pos	19.59	pos
O	BHU 51/2002	36.38	pos	No T_p	neg ^a
O	ETH 2/95	26.12	pos	26.61	pos
O	GHA 5/93	26.64	pos	21.47	pos
O	GRE 1/96	No Ct	neg	46.59	pos ^b
O	HKN 19/2001	24.7	pos	25.16	pos
O	HKN 3/2003	29.53	pos	20.31	pos
O	HKN 8/2002	24.07	pos	18.1	pos
O	IRN 27/2003	21.32	pos	No T_p	neg ^a
O	IRQ 30/2000	24.23	pos	18.57	pos
O	IRQ 5/94	No Ct	neg	No T_p	neg ^c
O	KEN 5/2002	18.43	pos	16.91	pos
O	LAO 1/2003	29.84	pos	21.78	pos
O	LAO 17/2003	27.76	pos	18.39	pos
O	LAO 26/2003	28.09	pos	No T_p	neg ^a
O	LAO 29/2003	32.81	pos	No T_p	neg ^a
O	LAO 31/2003	24.46	pos	26.85	pos
O	MAY 1/2001	27.57	pos	No T_p	neg ^a

(continued)

Table 2 (continued)

FMDV Serotype	Strain	TaqMan (Ct)	Interpretation	RT-LAMP (T_p)	Interpretation
O	MAY 2/2004	17.2	pos	14.65	pos
O	MAY 5/2002	22.63	pos	20.62	pos
O	MAY 68/95	22.4	pos	16.39	pos
O	MYA 4/2002	20.79	pos	16.92	pos
O	MYA 7/2002	26.29	pos	18.57	pos
O	NEP 6/2003	29.65	pos	15.04	pos
O	NEP 46/95	19.8	pos	18.12	pos
O	PAK 1/2003	22.29	pos	25.06	pos
O	TUR 3/2002	29.43	pos	19.41	pos
O	TUR 5/2002	39.91	pos	30.36	pos
O	UGA 17/98	No Ct	neg	No T_p	neg ^c
O	VIT 13/2002	19.09	pos	17.26	pos
O	VIT 6/2002	17.87	pos	12.52	pos
C	PHI 9/94	31.58	pos	No T_p	neg ^a
C	PHI 4/94	33.39	pos	21.24	pos
C	PHI 1/88	No Ct	neg	15.16	pos ^b
C	SAU 5/94	30.36	pos	23.45	pos
C	NEP 35/96	31.80	pos	No T_p	neg ^a
Asia1	AFG 1/2001	37.17	pos	18.12	pos
Asia1	BAN 2/96	20.35	pos	17.21	pos
Asia1	TUR 3/2000	20.19	pos	18.21	pos
SAT1	NIG 20/75	25.60	pos	No T_p	neg ^a
SAT1	TAN 43/99	21.13	pos	21.07	pos
SAT1	UGA 47/71	26.50	pos	16.98	pos
SAT1	ZAM 25/80	25.21	pos	33.28	pos
SAT2	ERI 7/98	21.43	pos	24.11	pos
SAT2	ETH 4/91	23.91	pos	21.19	pos
SAT2	KEN 7/99	16.64	pos	No T_p	neg ^a
SAT2	LIB 1/2003	33.11	pos	25.81	pos
SAT2	MOZ 22/78	19.45	pos	19.5	pos
SAT2	MOZ 22/78	34.13	pos	No T_p	neg ^a
SAT2	RHO 10/80	33.44	pos	30.65	pos
SAT2	SAR 11/83	25.71	pos	27.54	pos
SAT2	SAR 4/77	26.07	pos	32.8	pos
SAT2	SAR 4/77	28.32	pos	28.03	pos
SAT2	TAN 1/99	22.18	pos	28.58	pos
SAT2	ZIM 1/97	27.25	pos	No T_p	neg ^a
SAT2	ZIM 3/97	33.12	pos	17.93	pos
SAT2	KEN 2/02	19.37	pos	15.84	pos
SAT2	SAU 2/00	26.82	pos	15.23	pos
SAT2	SAU 6/00	17.80	pos	18.74	pos
SAT2	ZIM 1/04	23.04	pos	22.71	pos
SAT3	RHO 2/74	26.90	pos	33.42	pos
SAT3	SAR 12/80	26.52	pos	25.05	pos
SAT3	ZIM 2/84	33.96	pos	11.51	pos
SAT3	BEC 4/64	28.38	pos	49.89	pos
SVD*	UKG 4/93	No Ct	neg	No T_p	neg

neg^a = positive by TaqMan[®], negative by RT-LAMP (n = 13); pos^b = negative by TaqMan[®], positive by RT-LAMP (n = 2); neg^c = negative by both TaqMan[®] and RT-LAMP (n = 4); pos = positive; *: Swine vesicular disease virus

starting template (10 copies), the amplification could only be detected by current diagnostic real-time PCR after 50 min (see Fig. 4B).

Evaluation of performance on reference virus strains

The use of a real-time thermocycler enabled up to 96 reactions to be monitored concurrently, allowing us to analyse a large number of representative FMDV isolates. In initial experiments, the performance of the RT-LAMP primers was evaluated using RNA prepared from cell cultured isolates representing all topotypes from each of the seven serotypes of FMDV. The TaqMan[®] assay was performed alongside the real-time RT-LAMP assay on the same set of RNA extractions from the reference samples. RT-LAMP was found to be capable of strong amplification of FMDV RNA prepared from all topotypes belonging to all serotypes of FMDV (Fig. 5).

Evaluation of performance on field virus samples

A more detailed evaluation of the assay was performed using RNA extracted from clinical samples obtained from suspect cases of FMD and compared with TaqMan[®] real-time RT-PCR. In this study, RT-LAMP detected 81/98 of the field samples tested (Table 2). Three virus isolates (A BHU 35/2003, O GRE 1/96 and C PHI 1/88) previously designated as negative by TaqMan[®] were detected by RT-LAMP using specific primers. In contrast, thirteen samples designated positive by TaqMan[®], were not amplified by RT-LAMP.

Discussion

During the devastating outbreak of FMD in the UK in 2001 there was a 24/48 h cull policy, which meant that the majority of livestock on infected and contiguous premises were condemned on the basis of clinical presentation alone. However, clinical diagnosis is not completely accurate, especially in sheep, which were the main livestock species affected in 2001 [7]. As well as a requirement that diagnostic tests to confirm clinical suspicions should be more rapid and preferably performed on-site, new methods must be highly sensitive because of the serious consequences of false negative results. RT-PCR has been shown to be a powerful, sensitive and robust tool for FMDV detection [19, 20], but transferring RT-PCR to the field demands that sophisticated instrumentation be operated in less than perfect conditions. The RT-LAMP assay reported here is carried out in a single tube, incubating the mixture at a constant 65 °C for less than an hour in a standard waterbath or heat block. The advantages of the method are due to its simple operation, rapid reaction, and potential for visual interpretation without instrumentation. This could make the technique far more suitable for field deployment.

LAMP has been reported to be capable of detecting as little as 6 copies of starting template [15, 16], and RT-LAMP has been shown to be 10 times more sensitive than end-point RT-PCR [16]. The real-time RT-LAMP assay for FMDV has a dynamic range over nine orders of magnitude. The assay can detect 10 copies

of RNA template, which is equal to real-time RT-PCR and between 10 and 100 times more sensitive than orthodox RT-PCR (data not shown). The specificity of RT-LAMP is reportedly high [17] as it uses 4 primers targeted at 6 regions of DNA, or 6 primers for 8 targets when 'accelerated' LAMP is performed [16]. However, RT-LAMP outperformed real-time RT-PCR (TaqMan[®]) in terms of reaction speed. Ten copies of RNA template could be amplified by RT-LAMP to produce a visible result in 20 min; 10¹⁰ copies were detected in less than 8 min. In comparison, 10 copies of template were detected by the TaqMan[®] assay in 55 min. We have also shown that the RT-LAMP assay may be performed in the laboratory on a real-time thermocycler using 96-well reaction plates, enabling a high throughput of samples.

Real-time RT-LAMP using primers designed in the 3D region of FMDV generated positive results with field samples that comprised all 7 FMDV serotypes and included some samples that were previously designated as negative by TaqMan[®] RT-PCR. However, thirteen samples giving a positive reaction by TaqMan[®] were not detected by RT-LAMP. These results are not surprising due to the highly variable nature of the RNA genome of FMDV. During a recent validation exercise [12] it was revealed that several field isolates were not detected by either current diagnostic TaqMan[®] RT-PCR assays [3, 18] due to mismatches at the primer/probes sites. The failure of the RT-LAMP assay to detect certain isolates is also most probably due to sequence changes at specific sites within the primers. Although the current FMD-specific RT-LAMP primers amplify despite the presence of certain sequence mismatches, a mismatch at the 3' end of F1 and/or the 5' end of B1 would totally inhibit the loop cycling, a feature that enables the technique to be used in SNP analysis [11]. Further development of this 'first generation' FMDV assay will aim to identify mismatches with existing primers and to increase the sensitivity of the RT-LAMP assay by substituting nucleotides at these sites with synthetic bases (e.g. Inosine) or using a cocktail of primers that incorporate all possible nucleotides at these positions. The introduction of redundancy into LAMP primer design has not yet been formally demonstrated, but is currently being investigated in an attempt to overcome this problem with specificity.

It has been reported that unlike RT-PCR, RT-LAMP is not inhibited by contaminants carried over from the RNA isolation procedure and can be performed after relatively crude sample extraction procedures [8]. This report concerned the detection of viruses in plant tissue and so it must be determined whether crude extracts from animal samples, such as vesicular fluid and epithelium, blood, and oesophagopharyngeal fluid will have an inhibitory effect. Existing nucleic acid extraction methods are too elaborate for field use, and simpler procedures are needed. Since visualisation of a positive RT-LAMP result can be achieved by eye and denaturation of the template is not necessary, amplification may be carried out in a simple water bath or disposable thermal device or heat pack for field use. The possibility to use disposable equipment for this assay would overcome difficulties in decontaminating apparatus that has to be transferred between premises. An extremely rapid and sensitive test such as RT-LAMP that avoids the use of delicate and expensive instruments could therefore be invaluable to field diagnosis.

RT-LAMP may prove a cost-effective method for the detection of FMDV genomes at the pen-side, in the field, and by developing countries.

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