Rapid, sensitive and effective diagnostic tools for foot-and-mouth disease virus in Africa

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Speed is paramount in the diagnosis of highly infectious diseases, such as foot-and-mouth disease (FMD), as well as for emerging diseases; however, simplicity is required if a test is to be deployed in the field. Recent developments in molecular biology have enabled the specific detection of FMD virus (FMDV) by reverse-transcription loop-mediated isothermal amplification (RT-LAMP), real-time reverse-transcription polymerase chain reaction (RT-qPCR) and sequencing. RT-LAMP enables amplification of the FMDV RNA-dependent RNA polymerase 3D(pol) gene at 63 °C (in the presence of a primer mixture and both reverse transcriptase and *Bst* DNA polymerase) for 1 h, whilst RT-qPCR amplifies the same gene in approximately 2 h 30 min. In this study, we compared the sensitivity and effectiveness of RT-LAMP against RT-qPCR for the detection of the FMDV 3D(pol) gene in 179 oesophageal-pharyngeal scraping samples (collected by probang) obtained from clinically healthy cattle and buffalo in Malawi, Mozambique and Tanzania in 2010. The FMDV detection rate was higher with RT-LAMP (30.2%; $n = 54$) than with RT-qPCR (17.3%; $n = 31$). All samples positive by RT-qPCR (Cq $\leq$ 32.0) were also positive for the RT-LAMP assay; and both assays proved to be highly specific for the FMDV target sequence. In addition, the VP1 sequences of 10 viruses isolated from positive samples corresponded to the respective FMDV serotypes and genotypes. Our findings indicate that the performance of RT-LAMP is superior to RT-qPCR. Accordingly, we consider this test to have great potential with regard to the specific detection and surveillance of infectious diseases of humans and animals in resource-compromised developing countries.

Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease that affects cloven-hooved animals such as cattle, sheep, goats and pigs. FMD outbreaks have occured worldwide, resulting in significant economic losses (Knowles & Samuel 2003). Early identification of FMD virus (FMDV) is, therefore, critical for the control of disease and to minimise losses that could occur in livestock. Rapid and accurate diagnosis of FMDV is required for effective disease control. FMD cannot be distinguished clinically from other vesicular diseases, such as swine vesicular disease (SVD), vesicular exanthema of swine (VES) and vesicular stomatitis (VS); similarities that can pose challenges for early confirmation of field outbreaks. Routine laboratory diagnosis of FMD can be performed by a combination of antigen-capture enzyme-linked immunosorbent assay (ELISA) and virus isolation (OIE 2012). Antigen-capture ELISA only takes 4 h to perform; however, this test is only suitable for epithelium samples and does not have the analytical sensitivity required to confirm the negative status of premises (OIE 2012; Reid *et al.* 2001). In contrast, viral isolation is highly sensitive, but can take long time: up to two weeks to deliver a definitive answer. Several reverse-transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR (RT-qPCR) assays have been developed for the detection of FMDV (Callahan *et al.* 2002; Reid *et al.* 2000, 2001). RT-qPCR assays, which are highly sensitive and rapid and do not require electrophoresis, are now used widely for routine diagnosis of FMDV (Reid *et al.* 2009; Shaw *et al.* 2007). However, because these assays require a precision thermal cycler with a fluorescence detector and commercially-available kit-form reagents, these assays can be expensive to perform. Given these limitations, a rapid, simple, sensitive, and more cost-effective assay for diagnosis of FMD in clinical samples is required.

A recently-developed novel nucleic acid amplification method, termed loop-mediated isothermal amplification (LAMP) (Nagamine, Hase & Notomi 2002), is a promising candidate for rapid and easy detection of FMDV cDNA. LAMP is based on the principle of autocycling strand displacement

DNA synthesis performed by the large fragment of *Bst* DNA polymerase. A combined mix of AMV reverse transcriptase and *Bst* DNA polymerase enables simultaneous reverse transcription and DNA amplification to be undertaken in the same tube. Reverse-transcription loop-mediated isothermal amplification (RT-LAMP) assays are known to be more sensitive than conventional gel-based reverse-transcription polymerase chain reaction (RT-PCR) assays. In addition, they are fast and easy to perform since they require only a simple incubator, such as a heating block or a water bath, to provide a constant temperature for the reaction (Parida *et al.* 2006).

The aim of this study was to assess the performance of RT-qPCR and RT-LAMP assays for detection of the RNA-dependent RNA polymerase 3D(pol) gene of the FMDV genome from oesophageal-pharyngeal (OP) scraping samples (collected by probang) obtained from cattle and buffalo for the purposes of active surveillance.

Research method and design

Samples

Oesophageal-pharyngeal scrapings were collected from clinically healthy cattle and buffalo in Malawi (Lengwe National Park [NP]), Mozambique (Marromeu NP) and Tanzania (Katavi NP) in 2010. The samples were collected in separate vials containing transport media, then were transported to the Food and Agriculture Organization (FAO) World Reference Laboratory for FMD (WRLFMD) at Pirbright and stored at -20 °C until use.

RNA extraction

Total RNA was extracted from 600 µL elution buffer–sample mixture (140 µL original OP sample mixed with 460 µL elution buffer) using the RNeasy 96 BioRobot 9604 Kit (catalogue number 967142) on an automated robotic platform (MagNA Pure LC, Roche). The resultant total RNA was eluted in 50 µL nuclease-free H_2O and stored in aliquots of 10 µL at -80 °C until required.

Real-time RT-PCR assay

An established one-step RT-qPCR specific for the 3D(pol) region of FMDV (Reid *et al.* 2009, adapted from Callahan *et al.* 2002) was used in this study.

This one-step real-time RT-PCR assay was performed (in duplicate) using an Mx3005P qPCR system (Stratagene, La Jolla, CA, USA). Briefly, each 25 µL PCR reaction contained 0.8 µM each of the 3DF/3DR primers (100 µM stock concentration, Applied Biosystems), 0.3 µM 3DP TaqMan probe (labelled with FAM and TAMRA as reporter and quencher, respectively) and 0.5 µL Superscript III/Platinum Taq enzyme mix (supplied with the SuperScript® III Platinum® One-Step qRT-PCR Kit; catalogue number 11732-088, Life Technologies, Paisley UK) to amplify 5 µL of template RNA. The amplification involved incubation of reverse transcription reactions at 48 °C for 45 min, then a 10 min denaturation at 95 °C, followed by 50 cycles of 15 s for denaturation at 95 °C and 60 s combined annealing/extension at 60 °C. Following amplification, quantification cycle (Cq) values were assigned and the average Cq value of the results from duplicate samples was used for analysis. The primers and probe used are shown in Table 1.

RT-LAMP assay

Multiplex RT-LAMP assays were performed in a PCR reaction tube (PCR snap strip 0.2 mL Natural, catalogue number 3240-00, Anachem, Luton, UK) as described previously (Yamazaki *et al.* 2012). Briefly, a 25 µL volume reaction mix that contained 2 µL template RNA, 0.15 U AMV reverse transcriptase (Invitrogen, Carlsbad, CA, USA), 8 U *Bst* DNA polymerase (Large Fragment; New England Biolabs, Ipswich, MA, USA), 20 mM Tris-HCl (pH 8.8, Cambridge Bioscience, Cambridge, UK), 10 mM KCl (Sigma-Aldrich), 8 mM $MgSO_4$ (New England Biolabs), 10 mM $(NH_4)_2SO_4$ (Sigma-Aldrich), 0.1% Tween20 (Sigma-Aldrich), 0.8 M Betaine (Sigma-Aldrich), 1.4 µM each dNTP (GE Healthcare, Little Chalfont, UK), 1.6 µM each inner primer (8-FIP and 8-BIP), 0.2 µM each outer primer (81-F3 and 81-B3) and 0.8 µM each loop primer (81-LF and 81-LB). Each RT-LAMP reaction was incubated at 63 °C for 1 h, followed by 80 °C for 5 min. Amplified products were detected at 650 nm using a Loopamp EXIA turbidimeter (Teramecs, Kyoto, Japan). A reaction was considered positive when the differential value of the turbidity reached 0.1 FTU within 1 h. A Tp value (time of positivity, in minutes) was designated as the time at which this differential value reached this threshold. The evaluation of the multiplex RT-LAMP assays was performed using blind coded samples.

TABLE 1: Primers and probes used for one-step RT-qPCR and RT-LAMP for the detection of FMDV.

Target region for primers and probes	Type of primer or probe	Oligo name	Sequence (5' – 3')	Working conc (µM)	Gene location
FMDV 3D†	Forward primer	Callahan 3DF	ACTGGGTTTTACAAACCTGTGA	0.8	n/a
	Reverse primer	Callahan 3DR	GCGAGTCCTGCCACGGA	0.8	n/a
	TaqMan probe	Callahan 3DP	FAM-TCCTTTGCACGCCGTGGGAC-TAMRA	0.5	n/a
FMDV LAMP‡	Forward primer	81-F3	GTTGCAACCCTGATGTTGA	0.2	7331–7349
	Reverse primer	81-B3	GGCATGCTCCGTGTTCAC	0.2	7536–7519
	Forward inner primer	8-FIP	GTTCATCGCGTCACTGCAGTGTGCTCAGTACAGAAACGTGTG	1.6	7446–7426, 7374–7394
	Reverse inner primer	8-BIP	ATGTTTGAGGAAGTGTTCCGCACAGAGTCTTCAGGATCCACTC	1.6	7450–7472, 7517–7498
	Loop primer	81-LF	CATCAAAGGCCGAATAGTCCAC	0.8	7420–7399
	Loop primer	8-LB	GACTTCGGCTTCCACCC	0.8	7474–7490

Source: †Callahan *et al.* (2002); ‡Yamazaki *et al.* (2012)
RT, reverse transcriptase; PCR, polymerase chain reaction; FMDV, foot-and-mouth disease virus; LAMP, loop-mediated isothermal amplification; n/a, Not applicable.

Specificity of RT-LAMP assay

The RT-LAMP assay specificity was assessed using samples positive for swine vesicular disease virus (SVDV), vesicular stomatitis virus (VSV) and vesicular exanthema of swine virus (VESV) that cause infections that are clinically indistinguishable from FMD.

Results

A total number of 179 OP samples were tested for the 3D(pol) region of the FMDV genome by both RT-qPCR and RT-LAMP assays. Thirty-one samples (17.3%) tested positive for FMDV by RT-qPCR whilst 54 samples (30.2%) were positive for FMDV by RT-LAMP. The detection rate of FMDV was, on average, three to four times higher in samples collected from buffalo compared with those obtained from cattle in the livestock-wildlife interface areas of Katavi, Lengwe and Marromeu National Parks in Tanzania, Malawi and Mozambique respectively. The summary of results is shown in Table 2.

The multiplex RT-LAMP assay demonstrated higher sensitivity (54/179) in detection of FMDV RNA from OP probang scraping samples than RT-qPCR (31/179). All samples that produced a positive result by RT-qPCR (with Cq values ≤32.0) were positive by RT-LAMP assay. The 23 samples that tested positive by RT-LAMP but negative by RT-qPCR had Cq values ranging from 32.5 to 38.5. The RT-LAMP assay was shown to be specific for FMDV as all the RNA samples positive for SVDV, VSV and VESV were negative for FMDV. In addition, 10 samples that were positive for FMDV by both RT-LAMP and RT-qPCR were sequenced for the VP1 coding region and all sequences corresponded to the respective serotypes and/or genotypes of the virus.

Ethical considerations

This study was conducted in accordance with relevant national and international guidelines on handling animals and the ARRIVE guidelines (Kilkenny *et al.* 2010) for reporting *in vivo* animal experiments were adhered to.

Trustworthiness

The experiment was considered to be both reliable and valid. Reliability of the study was tested by repeating the same procedure for RT-LAMP and RT-qPCR on positive control RNA obtained from the reference FMDV isolate O1 Manisa (TUR/8/69), which yielded the same results on repeated

trials. The experimental procedures were performed with care and interpretation of results was done according to the established standards.

Discussion

Rapid, sensitive and specific detection methods for FMDV are necessary for deploying rational control methods for FMD in a specific geographic region. RT-qPCR and RT-LAMP are two sensitive and rapid diagnostic methods for FMDV. In contrast to RT-PCR (either real time or gel-based), the design of RT-LAMP assays is particularly challenging because a larger number of oligonucleotides is required in order to perform the test. In this study, the performance of RT-qPCR and RT-LAMP for FMDV detection on OP samples was evaluated. The performance of RT-LAMP was higher (30.2%; *n* = 54) than RT-qPCR (17.3%; *n* = 31) (Table 2). Several studies have shown that the diagnostic sensitivity of RT-LAMP assays for FMDV is high, ranging from 82.7% – 98.5% when tested against material comprising several FMDV serotypes (Chen *et al.* 2011; Dukes, King & Alexandersen 2006; Shao *et al.* 2010). The findings of this study indicate that RT-LAMP is relatively more sensitive than RT-qPCR in the detection of the FMDV 3D(pol) gene.

The RT-LAMP assay used in this study amplified the 3D(pol) region for 1 h at 63 °C whereas real-time RT-PCR took almost 2 h and 30 min in a thermocycler. The fact that RT-LAMP takes less time and does not necessarily require a thermocycler implies that there is high potential for its application in the field and in areas where specialised laboratory equipment is not available.

The OP samples analysed during this study were collected from clinically health cattle and African buffalo in livestock–wildlife interface areas. It has been reported previously that FMDV detection rates in OP samples are usually low in persistently-infected animals (Vosloo *et al.* 1996). The sensitivity of RT-LAMP with regard to detecting cases of FMDV that were negative by RT-qPCR (Cq values 32.5–38.5) raises the possibility that the RT-LAMP could be suitable for the identification of animals with persistent FMDV infections and could thus be applied to the surveillance of infectious diseases in Africa in the regions where they are known to be endemic.

As most countries in Africa are planning to adopt a strategic control programme for FMD through vaccination and controlled animal movements, a wider knowledge and

TABLE 2: Summary of results for oesophageal-pharyngeal samples tested during this study.

Location	Animal species	Number tested	RT-qPCR positive	RT-LAMP positive
Tanzania (Katavi NP)	Buffalo	31	15	18
	Cattle	30	3	5
Malawi (Lengwe NP)	Buffalo	30	4	9
	Cattle	30	0	3
Mozambique (Marromeu NP)	Buffalo	29	7	14
	Cattle	29	2	5
Total	**n/a**	**179**	**31 or 17.3%**	**54 or 30.2%**

NP, National Park; n/a, not applicable.

understanding of the dynamics and epidemiology of FMDV infection should be taken into consideration. This will require the rapid identification of high-risk 'hotspots' as well as potentially-infected and FMD-free zones. This task will, however, need the development of simple and cost-effective diagnostic tools such as RT-LAMP that can be deployed under field conditions in order to identify FMDV infection and to define the spatiotemporal distribution of virus serotypes in the region.

Previous studies have shown the heterogeneity of FMDV serotypes in Southern Africa (Kasanga *et al.* 2012; Vosloo *et al.* 2002). This heterogeneity highlights the need for continuous surveillance of FMD using rapid, simple and cost-effective methods that are able to discriminate serotypes and/or subtypes. The RT-qPCR and RT-LAMP assays evaluated during this study could not discriminate serotypes and/or subtypes, indicating that further research is required in order to develop RT-qPCR and RT-LAMP assays for this purpose, which could then be used in areas of high FMDV heterogeneity.

Limitations of the study

Possible limitations that could affect the results of the current study include the storage condition(s) of the OP samples and the time taken to transport the samples to the FAO World Reference Laboratory for FMD where the analysis was performed.

Recommendations

Further in-depth studies are required in order to evaluate the detection ability of RT-LAMP and RT-qPCR on field samples and to compare the performance of the two assays under different conditions such as temperature and moisture.

Conclusion

In conclusion, we have evaluated the performance of RT-qPCR and RT-LAMP assays in the detection of the 3D(pol) region of the FMDV genome from OP probang scrapings. We found that RT-LAMP was superior to RT-qPCR in the detection of FMDV from OP probang samples and could be used for surveillance of FMD in cattle and buffalo in developing African countries.

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Competing interests

The authors declare that they have no financial or personal relationship(s) that may have inappropriately influenced them in writing this article.

Authors' contributions

C.J.K. (Sokoine University of Agriculture) participated in the study design, the experimental work, the analysis and interpretation of the data and drafted the manuscript. W.Y. (University of Miyazaki), V.M (The Pirbright Laboratory) and D.P.K. (The Pirbright Laboratory) participated in the study design, the experimental work and drafting of the manuscript. M.M., J.D., C.M., P.C., L.J. (all Southern African Development Community) and E.R. (Ministry of Livestock Development and Fisheries) participated in sample collection and the writing of the manuscript. P.N.W and M.M.R (both Sokoine University of Agriculture) participated in the study design and the writing of the manuscript. All authors read and approved the final manuscript.

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