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Development of a Rift Valley fever real-time RT-PCR assay that can detect all three genome segments

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Outbreaks of Rift Valley fever in Kenya, Madagascar, Mauritania, and South Africa had devastating effects on livestock and human health. In addition, this disease is a food security issue for endemic countries. There is growing concern for the potential introduction of RVF into non-endemic countries. A number of single-gene target amplification assays have been developed for the rapid detection of RVF viral RNA. This paper describes the development of an improved amplification assay that includes two confirmatory target RNA segments (L and M) and a third target gene, NSs, which is deleted in the Clone 13 commercial vaccine and other candidate vaccines. The assay also contains an exogenous RNA control added during the PCR setup for detection of amplification inhibitors. The assay was evaluated initially with samples from experimentally infected animals, after which clinical veterinary and human samples from endemic countries were tested for further evaluation. The assay has a sensitivity range of 66.7–100% and a specificity of 92.0–100% depending on the comparison. The assay has an overall sensitivity of 92.5%, specificity of 95% and a positive predictive value of 98.7%. The single-tube assay provides confirmation of the presence of RVFV RNA for improved confidence in diagnostic results and a "differentiate infected from vaccinated animals" (DIVA) – compatible marker for RVFV NSs – deleted vaccines, which is useful for RVF endemic countries, but especially important in non-endemic countries.

1. Introduction

Recent outbreaks of Rift Valley fever (RVF) in Kenya (Nguku et al., 2010), Madagascar (Carroll et al., 2011), South Africa (Archer et al., 2011) and Mauritania (El Mamy et al., 2011) have highlighted the need for rapid and reliable diagnostic tools for detecting this zoonotic disease of significant public health, veterinary and socio-economic importance. Rift Valley fever virus (RVFV), a mosquito-borne Bunyavirus, is enzootic in sub-Saharan Africa with RVFV epidemics occurring at irregular intervals primarily in eastern and southern Africa (Gerdes, 2002). There are agricultural and public health concerns for the potential intentional or unintentional introduction of RVFV into non-endemic countries (Borio et al., 2002; Sidwell et al., 1994; Weaver and Reisen, 2010). The ability of arboviruses to rapidly establish themselves in new ecosystems was exemplified by the introduction of West Nile virus to North America (Roehrig et al., 2002). Disease outbreaks vary significantly in the percentage of susceptible animals affected, ranging from 15% to 90% (EMPRES, 2005). Those which succumb display severe disease, coupled with abortion in pregnant cattle, sheep, and goats and 70–100% mortality in young animals (Gerdes, 2004). Endemic prevalence of antibody in humans ranges from 5 to 40% (EMPRES, 2005), but increases with risk factors such as livestock handling, exposure to aerosols during the slaughter of infected animals and consumption of raw milk (Gerdes, 2004). Infection of humans can result in hepatitis, hemorrhagic fever, encephalitis, ocular degeneration, and death (Al-Hazmi et al., 2003; Archer et al., 2011; Gerdes, 2004).

Although veterinary vaccines are available, immunization of humans can only be accomplished through the use of an

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experimental inactivated RVFV vaccine with limited availability (Rusnak et al., 2011). Veterinary vaccines used in endemic and epidemic countries include a formalin-inactivated vaccine and an attenuated vaccine strain (Smithburne neurotropic strain) that was developed in 1949 (Gerdes, 2002; Smithburn, 1949). A naturally attenuated strain isolated from an asymptomatic human case in the Central African Republic, Clone 13, which has a large deletion in the open reading frame of the NSs protein, has been demonstrated to be safer than the Smithburn attenuated vaccine strain, which can cause abortions (Dungu et al., 2010; von Teichman et al., 2011). Clone 13 is now commercially available for use in livestock in South Africa. Other attenuated vaccine candidates have been investigated and preliminary results are promising, but these candidates are not yet licensed for human or animal use (Bird et al., 2008; Morrill and Peters, 2011) (for a recent review refer to Ikegami and Makino (2009)). An important feature of these newer veterinary vaccines is the ability to differentiate infected from vaccinated animals (DIVA). To employ a DIVA control strategy effectively, relevant companion diagnostic assays are required.

The RVFV genome consists of three negative sense, single-stranded RNA segments encoding structural and non-structural proteins. The large segment (L) encodes the RNA-dependent RNA polymerase which associates with the nucleocapsid protein (encoded by the small (S) segment) and genome segments to form ribonucleocapsids. The S-segment, which utilizes an ambisense coding strategy, additionally encodes a non-structural protein (NSs), which is the virulence factor of the virus that counteracts the host innate immune response. The medium (M) segment encodes two major envelope glycoproteins and two minor proteins, one of which is non-structural and may have anti-apoptotic properties (Won et al., 2006).

Diagnostic assays for detection of RVFV antibodies and antigen have been developed. Various enzyme-linked immunosorbent assays (ELISAs) have been developed for the detection of antibodies to RVFV in humans, sheep, cattle and wildlife species (Meegan et al., 1987; Paweska et al., 2005a,b, 2008; Williams et al., 2011). Antigen capture ELISAs are also available (Fukushi et al., 2012; Morvan et al., 1991; Jansen van Vuren and Paweska, 2009). Rapid RVFV RNA detection methods using real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) have also been reported (Bird et al., 2007a; Drosten et al., 2002; Garcia et al., 2001). In addition, a real-time reverse transcription loop-mediated isothermal amplification (LAMP) test for rapid detection of RVFV has been developed (Euler et al., 2012; Le Roux et al., 2009; Peyrefitte et al., 2008). Recently, methods for rapid inactivation of the virus and single-step rRT-PCR for detection of RVFV RNA were developed (Drolet et al., 2012). None of these tests are compatible or applied as a DIVA companion diagnostic assay. Furthermore, the need for detection of an introduced foreign animal pathogen is substantiated by its significant economic impact. Therefore, in this study a robust one-step quadruplex rRT-PCR assay was developed that allows for DIVA compatibility, detection confirmation, and exogenous internal control amplification.

2. Materials and methods

2.1. Viruses

RVFV MP-12 was propagated in fetal lung fibroblast (MRC-5) cell cultures. Propagation of RVFV strains from varying geographical and locations over 63 years was done in confluent African green monkey kidney epithelial cells (Vero). Cells were infected using 0.01 multiplicity of infection and RNA extractions were performed when approximately 80–95% of the infected cells showed cytopathology.

2.2. RNA extraction from infected cells or serum

Total RNA was extracted from cells using a variety of RNA isolation kits as per the manufacturer's protocols. The choice of extraction kit was based on local availability and/or preferences. RNA from RVFV propagated in cell culture was isolated using Trizol LS according to the manufacturer's recommendations (Life Technologies, Inc., Grand Island, NY). The primary method used for serum or tissue samples was the high-throughput RNA extraction method using the magnetic-bead capture kits: MagMAX-96 total RNA Isolation and MagMAX Viral RNA Isolation. Briefly, 130 μl of lysis/binding buffer was added to 50 μl of sample and mixed by shaking in a 96-well plate. Bead mix (20 μl) was then added and the mixture was shaken for 5 min. Four subsequent washes were performed (150 μl each) and the RNA was eluted in 50 μl of elution buffer at 65 °C. RNA was extracted both manually and automatically using available commercial kits in use at the various cooperating laboratories. The primary kit used in development of the assay was the Applied Biosystems MagMax Express system (Life Technologies, Inc., Grand Island, NY). RNA was quantitated using UV spectroscopy. RNA from veterinary clinical samples was purified using the MagNA Pure High Performance Total Nucleic Acid Isolation Kit together with the MagNA Pure LC according to manufacturers specifications (Roche Applied Science, South Africa).

2.3. Primer/probe design

The initial primer and probe design was based on previous real-time assays (Bird et al., 2007a; Drosten et al., 2002; Garcia et al., 2001). Subsequent new primer and probes were designed using Visual OMP (DNA Software, Ann Arbor, MI). The RVFV vaccine strain, MP-12, was used as a model virus for many of the studies due to lack of local access to an approved BSL-3+ laboratory for virulent RVFV. The L segment primer design was modified when MP-12 was used to account for sequence variation. The two exogenous internal control RNA primer and probe combinations were based on previously published assays (Drolet et al., 2012; Schroeder et al., 2012). The final primer design contained 4 primer sets and probes (Tables 1 and 2).

2.4. Optimization of new RVFV signatures for real-time RT-PCR (rRT-PCR)

The rRT-PCR procedure was performed as described previously (Wilson et al., 2009a,b). Optimization of the primer and probes were conducted individually, followed by multiplexing. Various quenchers and fluorescent dyes were evaluated using limit of detection (LOD) studies on the instruments available. The primary instrument used for a small number of samples was the Cepheid SmartCycler II (Cepheid Inc., Sunnyvale, CA), while for high-throughput the Agilent MX3005p (Agilent Technologies, Inc., Santa Clara, CA) was used. The initial evaluation of the primer probe designs was done using various plasmids containing RVFV target L, M and S sequences. For LOD experiments, samples were run in triplicate with viral RNA purified from 10-fold dilutions of RVFV MP-12 titered stock or in duplicate from a virulent RVFV titered stock. In some cases plasmids containing the target virulent RVFV sequences were used to facilitate optimization. Ct values were recorded and the mean and standard deviations calculated. Initial experiments were conducted with only the RVFV signatures and optimized using the iCycler (Bio-Rad, Hercules, CA). Two external RNA amplification controls were later added, optimized and evaluated on the Cepheid SmartCycler II.

Table 1
RVFV primers for real time RT-PCR.

Primer	Orient	Final conc. (μM)	T_m (°C)	Nucleotide sequence 5′–3′
RVFL-2912fwdGG[a]	Forward	10	53.1	TGA-AAA-TTC-CTG-AGA-CAC-ATG-G
RVFL-2981revAC	Reverse	10	52.7	ACT-TCC-TTG-CAT-CAT-CTG-ATG
RVFV-M(G2)-F(RVAs)	Forward	10	56.2	CAC-TTC-TTA-CTA-CCA-TGT-CCT-CCA-AT
RVFV-M(G2)-R (RVS)	Reverse	10	56.3	AAA-GGA-ACA-ATG-GAC-TCT-GGT-CA
RVFV-S(NSs)-F	Forward	20	55.7	TGA-TGG-TCC-TCC-CAG-GAT-AC
RVFV-S(NSs)-R	Reverse	20	55.8	ACT-AGG-ACG-ATG-GTG-CAT-GA
RVF-MP12-3296F[b]	Forward	10	53.6	CCT-CAC-TAT-TAC-ACA-CCA-TTC
RVF-MP12-3453R[b]	Reverse	10	50.5	ATC-ATC-AGC-TGG-GAA-GCT

[a] RVF L segment primers and probes identical to Bird et al. (2007a,b). RVF M primers and probes identical to Drosten et al. (2002).
[b] RVF-MP12 primers substituted for RVFL primers when RNA from RVFV MP-12 vaccine strain was used.

Table 2
RVFV probes for real time RT-PCR.

Probe	Fluorescent reporter dye (5′ end)	Quencher (3′ end)	Final conc. (μM)	T_m (°C)	Nucleotide sequence 5′–3′
RVFL-probe-2950	CAL FLUOR RED 610	BHQ2	10	62.9	CAA-TGT-AAG-GGG-CCT-GTG-TGG-ACT-TGT-G
RVFV-M(G2)	FAM	BHQ1	1	63.7	AAA-GCT-TTG-ATA-TCT-CTC-AGT-GCC-CCA-A
RVFV-S(NSs)	QUASAR 670	BHQ2	10	62.5	TCC-TGG-CCT-CTT-GGA-GAA-CCC-TC
RVF-MP12-3371P	CAL FLUOR RED 610	BHQ2	5	62	CTG-AGA-TGA-GCA-AGA-GCC-TGG-TTT-GTG-A

2.5. Real-time reverse transcriptase-polymerase chain reaction (rRT-PCR)

Final analysis was done after combining the RVFV triplex L, M and S primers and probes and one of the external RNA control combinations. The rRT-PCR was conducted using AgPath ID rRT-PCR Kit (Life Technologies, Inc., Grand Island, NY) and cycle times of 45 °C for 10 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min. Diagnostic test evaluation statistics was calculated (MedCalc Software, Mariakerke, Belgium).

3. Results

3.1. Multiplex rRT-PCR development and optimization

A one-step multiplex RT-PCR was initially developed based on previous real-time assays (Bird et al., 2007a; Drosten et al., 2002; Garcia et al., 2001). The primer-probe set targeting the S-segment did not perform consistently in the triplex assays. To further evaluate the design, alignments were made of all full-length sequences of the RVFV L, M and S RNA segments available from Genbank. Highly conserved regions were then identified for use in designing the real-time assay. These conserved segments were exported to VisualOMP and underwent further analysis for real-time development *in silico*. Further optimization was conducted piecewise with L, M and S *in vitro*, with plasmids containing half- to full-length genome segments from virulent strains of RVFV to determine feasibility for multiplexing. No significant sequence interactions were found after a BLAST search with the final designed primers. Plasmid DNA was serially diluted 6-fold and tested with various primers and probes individually and then multiplexed; multiple chemical and process parameters were evaluated during the optimization. After initial optimization with plasmid DNA, MP-12 total RNA was extracted from MRC5 cells at 80–95% CPE using Trizol LS following the manufacturer's protocol. This total RNA was serially diluted 6-fold and tested in the same fashion as for cDNA optimization. Optimal primer and probe set sequences can be found in Tables 1 and 2.

3.2. Quadruplex assay with evaluation of an exogenous armored enterovirus RNA control

Once the assay was optimized using the model system it was evaluated in laboratories in RVF endemic countries with authorization to work with virulent RVFV. The first design using the triplex for all three genome segments with different reporter dyes for each segment was evaluated in the Kenya Agricultural Research Institute (KARI). It was determined that the assay is able to detect Smithburn vaccine and Kenya 2007 outbreak strains of RVFV. The assay did not cross-react with Nairobi sheep disease virus that also causes hemorrhagic disease in ruminants. The condition of some of the samples clearly indicated that an external control was needed to control for RT-PCR inhibition. Previously, exogenous armored enterovirus RNA (Asuragen, Austin, TX) was successfully employed in a single-plex rRT-PCR assay (Drolet et al., 2012); therefore, it was decided to use the same control in this assay. The limit of detection of the quadruplex assay with viral RNA extracted from diluted titered stock of RVFV South African strain AR 20368 was 0.5 $TCID_{50/ml}$ (Fig. 1). No cross-reaction was found when the quadruplex assay was evaluated against a panel of nine other abortogenic or hemorrhagic viral agents (Table 3). In a separate experiment, a sample known to be positive for Nairobi Sheep disease virus also tested negative with the triplex real-time RT-PCR assay. The ability of the assay to detect RVFV strains from varying geographical locations isolated over a period spanning 63 years was evaluated (Table 4). The assay did not consistently detect the S segment when the exogenous armored enterovirus RNA control was used. To compensate for this problem at this time the triplex assay design (excluding armored enterovirus RNA, primer and probe) was used for further evaluation. Thirty positive and 15 negative human sera samples, tested at the National Institute for Communicable Diseases (NICD) in South Africa and confirmed by the previously validated reverse transcriptase loop-mediated amplification assay (RT-LAMP) (Le Roux et al., 2009), also

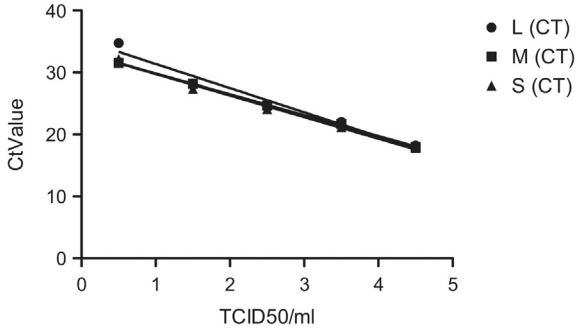

Fig. 1. Example of limit-of-detection analysis using RNA extracted from 10-fold dilutions of titered Rift Valley fever virus.

Table 3
Arboviruses tested that are not detected by the RVFV quadruplexrRT-PCR assay.

Virus	Family	Genus	Titer ($\log TCID_{50/ml}$)
Akabane	*Bunyaviridae*	*Orthobunyavirus*	6.8
Arumowot	*Bunyaviridae*	*Phlebovirus*	4.8
Chikungunya	*Togaviridae*	Alphavirus	7.5
Gabek Forest	*Bunyaviridae*	*Phlebovirus*	7.0
Gordil	*Bunyaviridae*	*Phlebovirus*	5.8
Saint Floris	*Bunyaviridae*	*Phlebovirus*	5.8
Dengue type I	*Flaviviridae*	*Flavivirus*	5.5
West Nile (lineage 1)	*Flaviviridae*	*Flavivirus*	7.8
Yellow Fever	*Flaviviridae*	*Flavivirus*	6.0

yielded identical results with the triplex format without an external RNA control (Table 5b).

3.3. Quadruplex assay with exogenous internal positive control (XIPC) control evaluation

An XIPC control (Schroeder et al., 2012) was substituted for the exogenous armored enterovirus RNA control, which provided more consistent results when run against RNA from samples from RVFV MP-12 infected cell-cultures and experimentally infected calves and lambs. This new quadruplex assay was also run against RVFV RNA extracted from sera of 25 calf, 27 goat and 41 sheep sera experimentally infected with RVFV ZH501 strain. All of these samples that were positive by the previously published monoplex rRT-PCR assay (Drolet et al., 2012) were also positive with the new quadruplex assay. The diagnostic specificity and analytical sensitivity with sample panels in Tables 3 and 4 were identical (Table 5a). The new quadruplex assay was evaluated at the ARC-Onderstepoort Veterinary Institute (South Africa) to compare it to the monoplex rRT-PCR Assay (Drosten et al., 2002) that was used for diagnosis of animal cases during the South African 2010 RVF outbreak. The assay was able to differentiate RNA from RVFV vaccine strain Clone 13 RNA from wild-type viral RNA. The sensitivity of the assay was poor, detecting only 78.4% of the field strain samples evaluated with the assumption that the original monoplex assay was correct. Of the 14 samples detected by the monoplex assay, but negative by the quadruplex assay, 4 had a positive detection of one segment only with the quadruplex assay. The samples not detected all had low amounts of viral RNA as evidenced by late Ct values when tested by the monoplex rRT-PCR. This low sensitivity was confirmed in most but not all by running individual segment monoplex real-time RT-PCR assays. The specificity in the analysis of veterinary clinical samples was 90.9%, resulting in an overall positive predictive value of 96.7%. The quadruplex assays with the XIPC control also indicated that 14% of the samples were negative for the NSs region of the RVFV S segment. This percentage of samples that the S target did not amplify is likely a reflection of the inclusion of samples from a recently vaccinated herd, probably with the NSs deletion Clone 13 vaccine strain.

The ability of the quadruplex assay using the XIPC control to detect the same RVFV strains and RNA extractions that was previously evaluated using the armored enterovirus RNA exogenous RNA control was determined. Contrary to the previous external

Table 5
Comparison of the RVFV quadruplex rRT-PCR to previously run monoplex rRT-PCR assays on clinical veterinary samples.

	1-Plex +'ve	1-Plex −'ve	Totals
a. RVF viral RNA positive samples from experimental infections of sheep, goats and calves			
4-Plex +'ve	93	0	93
4-Plex −'ve	0	0	0
Totals	93	0	93
Sensitivity 100%			
Specificity 100%			
Positive predictive value 100%			
	RT-LAMP +'ve	RT-LAMP −'ve	Totals
b. Human clinical samples			
4-Plex +'ve	30	0	30
4-Plex −'ve	0	15	15
Totals	30	15	45
Sensitivity 100%			
Specificity 100%			
Positive predictive value 100%			
	1-Plex +'ve	1-Plex −'ve	Totals
c. Veterinary clinical samples			
4-Plex +'ve	58	2	58
4-Plex −'ve	16	20	38
Totals	74	22	96
Sensitivity 75.7%			
Specificity 90.9%			
Positive predictive value 96.6%			
	1-Plex +'ve	1-Plex −'ve	Totals
d. Total clinical samples			
4-Plex +'ve	88	2	88
4-Plex–'ve	16	35	53
Totals	104	37	141
Sensitivity 84.6%			
Specificity 94.6%			
Positive predictive value 97.8%			

Table 4
Representative Ct values in detection of RVFV strains from various countries over 63 years.

Strain	Year of isolation	Source	Origin	No RNA control			With XIPC RNA control		
				L	M	S	L	M	S
Smithburn (UGA44)	1944		Uganda	13.2[a]	14.8	18.9	12.5	12.2	12.4
Lunyo UGA	1955	Mosquito	Uganda	13.7	12.2	12.3	17.0	15.4	17.0
B1143KEN77	1977		Kenya	12.3	12.1	12.2	12.6	12.1	12.4
ZH 501 EGY77	1977	Human	Egypt	12.9	12.4	13.9	14.1	13.8	13.3
ZH548 EGY77	1977	Human	Egypt	13.4	12.0	13.9	16.3	13.4	14.7
VRL2230/78	1978	Bovine	Zimbabwe	12.6	12.1	12.2	12.2	12.6	12.4
ArD38388BF83	1983	Mosquito	Burkina Faso	17.7	17.4	16.3	12.1	12.7	12.2
ArD3861SEN83	1983	Mosquito	Senegal	12.3	12.5	12.3	12.6	12.1	12.5
SPU384001KEN97	1997		Kenya	12.3	12.0	13.5	12.2	12.2	13.4
AR 21229	2000		Saudi Arabia	12.3	12.6	12.8	12.1	12.7	12.2
RVF 117/06	2006	Human	Kenya	23.9	17.5	nd[b]	12.8	12.4	12.3
AR 52/08	2008	Human	South Africa	12.6	12.8	12.5	12.8	13.4	12.8
SA 69/10	2010	Human	South Africa	14.6	14.6	15.5	14.7	15.1	15.2
SA579/11	2011		South Africa	12.1	12.1	12.5	12.5	12.4	12.9

[a] Numbers are Ct or cycle threshold values.
[b] nd: not detected.

control, the assay with the XIPC control did consistently detect the S-segment target. Overall, the quadruplex assay performed better with inclusion of the XIPC control when compared to using the armored enterovirus RNA control or the triplex format.

4. Discussion

The possible unintentional or intentional introduction of RVFV into a non-endemic country is of significant concern and it is therefore considered a high priority zoonotic disease (Chevalier et al., 2010; Hartley et al., 2011). Disease outbreaks begin in domestic livestock but often go undetected until human cases are confirmed. Therefore, there is a need for diagnostic tools that rapidly and specifically detect RVF virus in samples from infected animals. A number of assays are available for RVF diagnosis but many are not readily available in non-endemic countries (Wilson et al., 2013). Ideally, for non-endemic countries these assays should be safe to produce, store and handle in veterinary diagnostic laboratories. Genetic amplification assays, including real-time RT-PCR assays, meet these desired requirements. A sample handling protocol that quickly inactivates the potentially infected sample, yet facilitates nucleic acid extraction, leading to a single tube rRT-PCR assay that includes an internal exogenous RNA amplification control was developed previously (Drolet et al., 2012). The current rRT-PCR assays all detect a single region on the RVF viral genome. Although the RVFV genome is highly conserved (Bird et al., 2007b; Grobbelaar et al., 2011), genetic mutation that could affect the sensitivity of the assay always remains a possibility. The detection of RVF in a non-endemic country could have significant socio-economic implications. The assay design reported here is advantageous over previous assays in that it detects all three RVF viral genome segments, each with a different reporter dye. This design thus provides internal confirmation within a single tube assay. It is also useful in endemic countries that are using the licensed Clone 13 vaccine (von Teichman et al., 2011) in that the S segment target is within the deleted portion of this product. The ability to differentiate infected from vaccinated RVF viral RNA would have a limited time frame of effectiveness since the RNA persists from 2 to 6 days post infection or vaccination (Drolet et al., 2012). During the evaluation in South Africa there was an incident where vaccination had occurred just prior to observation of clinical disease in a sheep herd. In these samples this quadruplex analysis was found to be useful because it was able to establish that vaccine was present in some but also wild-type RVFV was present in other samples from this herd (data incorporated in overall evaluation). Thus wild-type infection had occurred too soon after vaccination to prevent clinical disease observed. The sensitivity of the assay depends significantly on the sample size and extraction protocol. When whole blood or serum samples were analyzed where the amount of material was not limited, the quadruplex assay was of identical sensitivity to standard assays currently in use. The tissue samples evaluated were from the 2010 South Africa RVF outbreak and were processed by one staff member who was vaccinated for RVF. To facilitate the sample handling process, swabs of the tissue sample were later used in many of the cases. In this case the amount of target RNA was more limited. The quadruplex assay was not as sensitive as the monoplex assay in detecting RNA in these samples (Drosten et al., 2002). All of the samples scored as “not detected”, had high Ct values >30, except for one with a value of >26 with the monoplex assay. These assays were done on frozen samples and 3 of the negative samples were also negative by monoplex as the time of the evaluation. It is not surprising that a quadruplex assay would have reduced sensitivity, but this can likely be improved by a small increase in the amount of extracted material used and/or improved extraction processes.

Two external RNA amplification controls were evaluated as useful additions to allow for the detection of PCR inhibitors. Both the armored enterovirus RNA (Drolet et al., 2012) and the XIPC controls (Schroeder et al., 2012) worked well with a majority of the samples and strains tested, however, the armored enterovirus RNA deleteriously affected the detection of the S segment target for a few strains. This may be due to run-to-run variations of the levels of S target. At the time of this analysis, the RNA from these samples was limited and further evaluation of these two external positive controls could not be re-run. The inconsistency with the armored enterovirus RNA was only noted in 30% of samples where the S target was detected in the triplex format (unpublished data). The design was limited to the NSs-encoding region of the RVF S viral genome segment, for DIVA compatibility with NSs-deleted attenuated vaccines. Four NSs primer sets were evaluated before a suitable pair was found to be effective and not to interfere with the L and M primers and probes. In future studies, the primer and probe sets targeting the L and M segments could be redesigned to reduce observed primer interaction, thus allow for a more consistent detection of the NSs target region. However, with the XIPC control the assay appeared to have improved specificity, detecting all three targets for all the strains evaluated. The L and M primer designs are from previously published procedures and are effectively being used in veterinary diagnostic laboratories currently. Therefore, to redesign the primer/probes at this time due to inconsistency with the enterovirus RNA control is not appropriate. The human clinical samples were run prior to the addition of the XIPC control. Unfortunately, these samples were no longer available to re-run using the current design. To obtain an overall estimate of the sensitivity using the available data, the veterinary and human data sets were combined resulting in an overall sensitivity of 82.7% and specificity of 94.6%. The overall evaluation of the assay was likely affected by the use of multiple RNA purification methods including both manual and automated procedures.

The triplex primer and probe design for all three genome segments of RVF virus can be utilized with a variety of instrument formats. The reporter and quenchers should be chosen according to the specifications of the instrumentants available. In cases where instruments do not have four-color channel capability, the L and M probes can utilize the same reporter dye using the S target as the confirmatory reporter for wild-type RNA. The quadruplex assay provides a more robust format with internal confirmation, potential DIVA-compatible RNA marker capability and the capacity to control for potential PCR inhibitors. An external RNA control can be used but in the evaluations the XIPC control had better specificity with the RVFV strains tested. Thus, the quadruplex assay is a useful new RVF viral genome detection tool for use in both endemic and non-endemic countries.

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