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Abstract (193 words)

In recent years, Madagascar and the Comoros archipelago have been affected by epidemics of Rift Valley fever (RVF), however detection of Rift Valley fever virus (RVFV) in zebu, sheep and goats during the post epidemic periods was frequently unsuccessful. Thus, a highly sensitive real-time RT-PCR assay was developed for the detection of RVFV at low viral loads. A new RVF SYBR Green RT-PCR targeting the M segment was tested on serum from different RVF seronegative ruminant species collected from May 2010 to August 2011 in Madagascar and the Comoros archipelago and compared with a RVF specific quantitative real time RT-PCR technique, which is considered as the reference technique. The specificity was tested on a wide range of arboviruses or other viruses giving RVF similar clinical signs. A total of 38 out of 2,756 serum samples tested positive with the new RT-PCR, whereas the reference technique only detected 5 out of the 2,756. The described RT-PCR is an efficient diagnostic tool for the investigation of enzootic circulation of the RVF virus. It allows the detection of low viral RNA loads adapted for the investigations of reservoirs or specific epidemiological situations such as inter-epizootic periods.

Keywords: Rift Valley fever virus; diagnostic tool; semi-nested RT-PCR; inter-epidemic period

1. Introduction

Rift Valley fever (RVF) is an arthropod-borne zoonotic disease affecting mainly domestic and wild ruminants as well as humans (Olive et al., 2012). Rift Valley fever virus (RVFV) is an enveloped RNA segmented virus belonging to the family *Bunyaviridae*, genus *Phlebovirus*, transmitted by a wide range of mosquito species with a tri-segmented single-stranded RNA genome of negative or ambisense polarity (S, M and L) (Suzich et al., 1990; Müller et al., 1994; Nichol et al., 2001; Schmaljohn and Hopper, 2001; Bird et al., 2007a; Turrell et al., 2008). Outbreaks have been reported in 19 countries from continental Africa, Indian Ocean islands (Madagascar, Union of the Comoros, Mayotte) and the Arabian Peninsula (Balenghien et al., 2013). Epidemiology of RVF is complex, associated with specific ecologic and climatic conditions such as heavy rainfalls that favor the emergence of a large number of adult mosquitoes maintaining RVF during interepidemic periods (IEP) through vertical transmission in *Aedes* vectors for Southern and Eastern Africa (Linthicum et al., 1985; Swanepoel and Coetzer, 2004). In addition, RVF isolations have been successful in forest-sampled mosquitoes in the Afro-Malagasy area, implicating wild vertebrates as potential reservoir hosts (Fontenille, 1989; Smithburn et al., 1948).

Whether the emergence of outbreaks in insular ecosystems is due to enzootic maintenance of the virus within the island or to importation from continental landmasses is unknown in the majority of cases (Tortosa et al., 2012). Recently, Bird et al. (2008) confirmed that RVFV activity can be detected at low levels in animal reservoirs during IEPs. In 2007, human RVF cases were noticed in 2 islands of the Comoros archipelago, Mayotte and Grande Comore (Sissoko et al., 2009).

Even if no case has been reported in this area since, seroconversion in animals has been observed in Madagascar and the Comoros archipelago including Mayotte, indicating persisting viral circulation in a post-epidemic period (Roger et al., 2009; Cêtre-Sossah et al.,

2012). Considering the proximity of these islands and the continuous animal trade between them, an active surveillance system for RVF was implemented between May 2010 and August 2011. The surveillance network targeting farm animal species was specifically designed to pick up active virus circulation in the context of the current post-epidemic phase in Madagascar and the Comoros archipelago. The implementation of a highly sensitive and specific diagnostic tool was a prerequisite for this large survey.

Techniques for the diagnosis of RVF include virus isolation, detection of specific antibody responses, and detection of the viral nucleic acids. Enzyme-linked immunosorbent assays, based on antigens or the recombinant nucleocapsid protein of RVFV have been extensively validated for the serodiagnosis of RVF (World Organisation for Animal Health, 2004; Paweska et al., 2005, 2008). Reverse transcriptase PCR (RT-PCR) assays are currently the most rapid and sensitive tests for the detection and quantification of RVFV. Conventional RT-PCR (Sall et al., 2002) and real-time RT-PCR (rtRT-PCR) based on TaqMan probe technology are the most used techniques during outbreaks (Garcia et al., 2001; Drosten et al., 2002; Bird et al., 2007b; Weidmann et al., 2008). Several studies have highlighted the highest analytical sensitivity obtained when using two-steps protocol RT-PCR detection (Sall et al., 2002). In order to improve the detection of very low concentrations of viral nucleic acids in samples taken during a RVF post-epidemic or inter-epizootic period, a highly sensitive, RVFV specific, real-time semi-nested RT-PCR detection system was developed. Specificity and sensitivity of the test was comparatively assessed with one of the currently available techniques used as reference. The new RT-PCR proved to be more adapted to detect low-level of RVF circulation in specific epidemiological conditions such as those in Comoros archipelago, Mayotte and Madagascar where seroconversions occur in the absence of clinical signs, and is therefore a useful tool for the study of RVF circulation during IEPs.

The main objective was to develop a highly sensitive real-time RT-PCR assay for the detection of Rift Valley fever virus.

2. Material and Methods

2.1. Cells, Viruses and samples

Vero cells were grown at 37°C in Dulbecco's modified medium (DMEM) (GIBCO, USA) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non essential amino-acids, 1% fungizone, 1% penicilin-streptomycin.

The following RVFV strains were used: the Smithburn vaccine strain obtained from the OBP (Ondersterpoort biological products, Onderstepoort, South Africa), four distinct Mauritanian RVF strains isolated during the 2010 outbreak (MT24010, MT25010, MT26010, MT28010) by the LNERV/ISRA laboratories respectively in Mauritania and Senegal and three RVF strains from Madagascar isolated during the 2008-2009 outbreak (776-08, 1464-08, 1585-08) by the Institut Pasteur, Madagascar.

The animal cohort (204 zebu in Madagascar, 104 zebu, sheep and goats in Mayotte and 182 zebu, sheep and goats in the Union of Comoros) was chosen because of their RVF seronegative status tested with a commercial ELISA kit (Comtet et al., 2010). The animals were bled monthly as part of an epidemiological survey held in a post-epidemic period from May 2010 to August 2011, giving a total of 2,756 serum samples. Serum samples were stored at -80°C.

2.2. RNA extraction

Viral RNA was extracted from 10 µl of either Smithburn strain infected Vero cell supernatant titrating to $10^{6.3}$ $TCID_{50}$/ml or from 200 µl of a pool of 5 serum samples and spiked with an internal control MS2 bacteriophage (Ninove et al., 2011) by using the EZ1 Virus Mini kit

v2.0 (Qiagen, Hilden, Germany) and the EZ1 robot, according to the manufacturer's protocol. Positive pools were analyzed individually with the aim of identifying the positive individual.

2.3. Primer design

The complete M RNA segments of 52 RVF virus isolates retrieved from GenBank were aligned via Muscle (Edgar, 2004) using the Geneious pro software package (v5.5) (Drummond et al., 2011). A highly conserved 110 bp genomic region within the RVF Gc-glycoprotein gene was identified matching 100% of the selected RVF isolates (nucleotides 2,656 to 2,766). This region was used for the design of the forward and reverse primers.

2.4. cDNA synthesis and real-time PCR conditions

cDNA templates were produced from a 20 µl RNA aliquot with 0.4 µg of random hexamers (Promega, Madison, USA) by reverse transcription using the GoScript kit (Promega, Madison, USA) in a final volume of 40 µl, as described by the manufacturer. The 2,756 samples collected during the epidemiological serosurvey were also analyzed without the step of reverse transcription to verify the absence of residual DNA.

The following primers listed in figure 1 were determined to be optimal: RVFV-M-2,656F, RVFV-M-2,840R (first round PCR) and RVFV-M-2,766R (second round semi-nested PCR). Several experiments highlighted that the highest analytical sensitivity could be obtained using a two-step RT-PCR protocol detection. To avoid cross-contamination, experiments were performed within an imposed one-way laboratory environment where amplified PCR products were never manipulated in the same area as the original samples. Additionally, individual tubes were used and multiple negative controls and no positive control were included in each PCR protocol. Amplification reaction mixtures were prepared in a final volume of 25 µl consisting of 12.5 µl GoTaq qPCR SYBR Green mastermix 2X (Promega, Madison, USA) and 5 µl of the nucleic acid matrix. For the first round PCR, 0.1 µM of each primer RVFV-M-2,656F and RVFV-M-2,840R was used while 0.4 µM of each primer, RVFV-M-2,656F and

RVFV-M-2,766R was used for the second round PCR. Thermal cycling for both amplification steps was performed in a Mx3005P real-time thermocycler (Stratagene, USA) as follows: 95°C for 5 min; 45 cycles of [95°C for 15 s, 60°C for 30 s and 72°C for 45 s]. A dissociation analysis was carried out following PCR to identify the right product by its specific melting temperature. The dissociation cycle was 95°C for 1 min, 60°C for 30 s and heating to 95°C at 0.1°C/s with continuous measurement of fluorescence. The expected sizes of these PCR products are 185 bp and 110 bp for the first and second round PCR, respectively.

2.5. Comparative efficiency

The performance of the new RT-PCR was compared with that of a Taqman RT-PCR considered as the current reference detection system (Bird et al., 2007b; Escadafal et al., 2013). The comparison was made by determining the detection limit of each assay using 10-fold serial dilutions (10^{-2} to 10^{-9}), prepared from the supernatant of Vero cells that had previously been infected by the Smithburn strain. The supernatant titrated to $10^{6.3}$ $TCID_{50}$/ml.

2.6. Analytical specificity of the new RT-PCR

RNAs from other vector-borne viruses were tested, as well as other viruses giving clinical signs similar to RVF, such as abortions or fever. These viruses are listed in Table 1.

2.7. Analytical sensitivity based on field samples

The sensitivity of the new RT-PCR was assessed on a bank of 2,756 serums collected from 490 zebu, sheep and goats that were serially bled in Madagascar and the Comoros archipelago and compared to the same Taqman RT-PCR (Bird et al., 2007b; Escadafal et al., 2013) and calibrated using a standard control based on the dilution range described above. Samples tested positive with the new RT-PCR were considered as true positives only after sequencing of the amplified material.

2.8. *Serological status of the RVF RT-PCR samples*

The serological status of the 490 animals was assessed monthly with a commercial ELISA kit (Comtet et al., 2010).

2.9. Sequence analysis

PCR products sequencing was performed by Beckman's Coulter Genomics, UK. Blast alignments were generated using MUSCLE through the Geneious pro software package (v5.5) (Biomatters Ltd, UK) (Edgar, 2004; Drummond et al., 2011).

2.10. Statistical analysis

Data were recorded and analyzed statistically with the R software package (version 3.0.1) (R Development Core team, 2009). Results with a two-sided p-value ≤ 0.05 were scored as being significant.

3. Results

3.1. *Primer design*

From the analysis of multiple sequence alignments of the M segment of 52 biologically and geographically diverse RVF virus strains, a highly conserved region was identified at positions 2511 to 2880 as illustrated in figure 1. Three distinct primers spanning this region were designed.

The nucleotide alignment and the position of the three primers are presented in Figure 1: two of the primers (RVFV-M-2656F, RVFV-M-2766R) gave a 100% match with all strains and the remaining primer named RVFV-M-2840R included 2 degenerated nucleotides (Fig. 1).

3.2. Comparative efficiency

Based on ten-fold serial dilutions of the Smithburn vaccine strain, the reference Taqman RT-PCR test (Bird et al., 2007b; Escadafal et al., 2013) was able to detect RVFV to a dilution of 10^{-3} whereas the new RT-PCR was able to detect to a dilution of 10^{-5} (Fig 2).

3.3. Analytical specificity

No false positive or false negative results were observed: All viruses belonging to different tested viral families (listed in Table 1) were negative using the new RT-PCR, and all PCR products obtained during screening gave the expected RVFV sequence in sequence analysis.

3.4. Analytical sensitivity based on field samples

All serum samples from the 2,756 serially bled zebu, sheep and goats were tested by the new RT-PCR and by the reference technique. A total of five samples tested positive by the reference RT-PCR, whereas 38 samples tested positive using the new RT-PCR, including all five detected using the reference technique. The new RT-PCR system, thus, demonstrated at least a 7.7-fold ($p=2.313e^{-7}$) higher sensitivity than the reference system (Table 2). Integration of RNA viruses into host genomes has previously been demonstrated (Katzourakis and Gifford, 2010), however, no PCR products were amplified in the absence of reverse transcription, thus excluding this as a possibility for tested RVFV PCR-positive animals.

3.5. Serological status of the RVF RT-PCR samples

Serum samples were available for consecutive months of sampling for 18 of the 38 RT-PCR positive animals. Seven of these animals seroconverted one month after sampling, whereas 11 animals did not (Table 3).

4. Discussion

Highly sensitive molecular assays have already been developed for the detection of RVFV based either on conventional PCR or on real-time PCR. These techniques have been shown to diagnose RVFV infection during epidemics. The specifically low viral loads associated with post-epidemic or inter-epidemic periods require extreme sensitivity and robustness from detection systems for the successful study of RVF circulation in potential reservoir animals during these epidemiological phases.

The presented RT-PCR proved to be both specific and sensitive and could detect 2 log dilutions further than the reference Taqman system (Bird et al., 2007b; Escadafal et al., 2013). Using 2,756 serum samples collected in the two countries as a test group, the reference technique detected only 5 positive samples. In contrast, the new RT-PCR allowed detection of 38 positive samples, corresponding to at least a 7.7 times higher sensitivity than the currently available gold-standard.

The new RT-PCR detection system performed well, even in the presence of mutations. On the forward-sense primer RVFV-M-2656F, 100 % of homology was observed among all strains and on the antisense primer RVFV-M-2766R, only one mutation was observed on the first nucleotide for 6 strains. Genomic diversity was assessed and showed the presence of 7 mutations in 2 sequences (CVII 14 and CVII 15) and 4 mutations for 2 sequences (ABMR 030 and ABMR 040), all isolated from Anjouan (Figure 3). The remaining sequences only presented erratic mutations. These results confirmed the strength of the primer designed in 2 conserved areas.

In 2007, the first cases of RVF were reported in the Comoros archipelago with evidence of animal seroconversions. During the epidemiological survey conducted between May 2010 and August 2011, the incidence of infection assessed by animal seroconversion was estimated at 14.4 % in the Union of Comoros in goats, sheep and zebu and 4.94 % in Madagascar in zebu. Out of the 18 positive animals detected by the new RT-PCR, 7 animals seroconverted with a specific RVF IgG antibody response, 11 did not seroconvert. The fact that a large proportion of animals that tested PCR positive did not show evidence of seroconversion to RVFV as assessed by Elisa is interesting and deserves comments. Firstly, the serology-based detection methods employed have inherent limits to the level of antibody that they can detect. More generally, the scale of the host immune response mounted after an infection is dependant on several host-associated factors including age, health, nutritional status and

immuno competence of the host. The pathogen infecting dose and its potential to actively replicate in dendritic cells are also likely to correlate with the magnitude of the invoked immune response, and therefore low-level responses may be expected during inter-epidemic periods, when viral loads are (on the whole) low. During epizootics, occasional animals may prove positive to the pathogen by molecular techniques but fail to show evidence of seroconversion or have delayed seroconversion. In a serosurvey of naturally acquired caprine arthritis encephalitis virus infection in goats, almost 20% of seronegative animals and apparently healthy animals had positive PCR test results and only half of them seroconverted by 8 months later (Rimstad et al., 1993). All these factors are likely operational in the field conditions characteristic of Madagascar and Comoros and may have implications to the epidemiology of RVFV; particularly, whether animals detected PCR positive but failed to seroconvert are contagious and participate to the low level transmission of RVF in the post epidemic period is presently unknown. The new RT-PCR allowed the detection of RVFV in 38 samples collected in a global epidemiological network in the Comoros archipelago and Madagascar considered as insular ecosystems (Tortosa et al., 2012). Both serological and molecular findings strongly suggest that the RVF virus is circulating in this area without inducing any clinical signs in infected animals.

During IEPs, the epidemiology of RVF remains unclear and involves a possible cryptic enzootic transmission cycle (Linthicum et al., 1985; Bird et al., 2008). Up to now, the detection of the RVF virus in RVF potential species reservoirs such as bats (*Roussetus obliviosus*, *Chaerephon pusillus*, *Miniopterus griveaudi*) in Comoros (n=96) and Maki catta (*Lemur catta*) in Mayotte (n=52) was unsuccessful (unpublished data), as well as in Madagascar (Olive et al.; 2013). The question of a possible inside virus propagation via an animal reservoir or a transitory animal host in the Indian Ocean area is still addressed. The

use of sensitive detection tools will be a great help in the understanding of the RVF inter-epidemic circulation.

Acknowledgments

The authors would like to thank the CRVOI team, La Réunion (Drs Roger and Girard, C. Foray) for their implication in the epidemiological survey. They also thank Dr D.A. Wilkinson for his comments and careful reading, all the field team in Madagascar (J. Rasolofoniaina, N. Andriamanampy, M.-M. Olive) the veterinarians (Drs Evenar, Rabenja, Remoro) and veterinarian assistants of Tulear I, Tulear II, Mampikony districts, the FOFIFA-DRZV (Drs J. Ravaomanana and R. Rakotondravao), Dr L. Bibias Armand and the Comorian partners (Mohamed Halifa) for their assistance during the collection campaign and P. Gil, for her help in lab experiments at CIRAD, Montpellier.

References

Balenghien, T., Cardinale, E., Elissa, N., Failloux, A.-B., Nipomichene, T.N.J.J., Nicolas, G., Rakotoharinome, V.M., Roger, M., Zumbo, B., 2013. Towards a better understanding of Rift Valley fever epidemiology in the south-west of the Indian Ocean. Vet. Res. 44, 78.

Bird, B.H., Khristova, M.L., Rollin, P.E., Ksiazek, T.G., Nichol, S.T., 2007a. Complete genome analysis of 33 ecologically and biologically diverse Rift Valley fever virus strains reveals widespread virus movement and low genetic diversity due to recent common ancestry. J. Virol. 81, 2805-2016.

Bird, B.H., Bawiec, D.A., Ksiazek, T.G., Shoemaker, T.R., Nichol, S.T., 2007b. Highly sensitive and broadly reactive quantitative reverse transcription-PCR assay for high-throughput detection of Rift Valley fever virus. J. Clin. Microbiol. 45, 3506-3513.

Bird, B.H., Githinji, J.W., Macharia, J.M., Kasiiti, J.L., Muriithi, R.M., Gacheru, S.G., Musaa, J.O., Towner, J.S., Reeder, S.A., Oliver, J.B., Stevens, T.L., Erickson, B.R., Morgan, L.T., Khristova, M.L., Hartman, A.L., Comer, J.A., Rollin, P.E., Ksiazek, T.G., Nichol, S.T., 2008. Multiple virus lineages sharing recent common ancestry were associated with a Large Rift Valley fever outbreak among livestock in Kenya during 2006-2007. J. Virol. 82, 11152-1166.

Cêtre-Sossah, C., Pédarrieu, A., Guis, H., Defernez, C., Bouloy, M., Favre, J., Girard, S., Cardinale, E., Albina, E., 2012. Prevalence of Rift Valley Fever among Ruminants, Mayotte. Emerg. Infect. Dis. 18, 972-975.

Comtet, L., Pourquier, P., Marié, J.L., Davoust, B., Cêtre-Sossah, C., 2010. Preliminary validation of the ID Screen® Rift Valley Fever Competition Multi-species ELISA. Poster presented at the 2010 EAVLD meeting, Lelystad, The Netherlands.

Drosten, C., Göttig, S., Schilling, S., Asper, M., Panning, M., Schmitz, H. Günther, S., 2002. Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, dengue virus, and yellow fever virus by real-time reverse transcription-PCR. J. Clin. Microbiol. 40, 2323-2330.

Drummond, A.J., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Duran, C., Field, M., Heled, J., Kearse, M., Markowitz, S., Moir, R., Stones-Havas, S., Sturrock, S., Thierer, T., Wilson, A., 2011. Geneious v5.4. Available from http://www.geneious.com/

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792-1797.

Escadafal, C., Paweska, J.T., Grobbelaar, A., le Roux, C., Bouloy, M., Patel, P., Teichmann, A., Donoso-Mantke, O., Niedrig, M., 2013. International External Quality Assessment of Molecular Detection of Rift Valley Fever Virus. PLoS Negl. Trop. Dis. 7, e2244.

1 Fontenille, D., 1989. Transmission cycles of arboviruses in Madagascar. Arch. Inst. Pasteur
2 Madagascar. 55, 7-317.
3 Garcia, S., Crance, J.M., Billecocq, A., Peinnequin, A., Jouan, A., Bouloy, M., Garin, D.,
4 2001. Quantitative real-time PCR detection of Rift Valley fever virus and its application to
5 evaluation of antiviral compounds. J. Clin. Microbiol. 39, 4456-4461.
6 Katzourakis, A. and Gifford, R.J., 2010. Endogenous viral elements in animal genomes. PLoS
7 genetics. 6, e1001191.
8 Linthicum, K.J., Davies, F.G., Kairo, A., Bailey, C.L., 1985. Rift Valley fever virus (family
9 Bunyaviridae, genus Phlebovirus). Isolations from Diptera collected during an inter-epizootic
10 period in Kenya. J. Hyg. (Lond). 95, 197-209.
11 Müller, R., Poch, O., Delarue, M., Bishop, D.H., Bouloy, M., 1994. Rift Valley fever virus L
12 segment: correction of the sequence and possible functional role of newly identified regions
13 conserved in RNA-dependent polymerases. J. Gen. Virol. 75, 1345-1352.
14 Nichol, S.T., 2001. Bunyaviruses, In: Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A.,
15 Martin, M.A., Roizman, B., Straus S.E. (Eds.), Fields virology, fourth ed. Lippincott,
16 Williams & Wilkins, Philadelphia, pp. 1603-1633.
17 Ninove, L., Nougairede, A., Gazin, C., Thirion, L., Delogu, I., Zandotti, C., Charrel, R.N., De
18 Lamballerie, X., 2011. RNA and DNA bacteriophages as molecular diagnosis controls in
19 clinical virology: a comprehensive study of more than 45,000 routine PCR tests. PLoS One 6,
20 e16142.
21 Olive, M.M., Goodman, S.M., Reynes, J.M., 2012. The role of wild mammals in the
22 maintenance of rift valley Fever virus. J. Wildl. Dis. 48, 241-266.

Olive, M.M., Razafindralambo, N., Andrianaivo Barivelo, T., Rafisandratantsoa, J.T., Soarimalala, V., Goodman, S.M., Rollin, P.E., Heraud, J.M., Reynes, J.M., 2013. Absence of Rift Valley fever virus in wild small mammals, Madagascar. Emerg. Infect. Dis. 19, 6.

Paweska, J.T., Mortimer, E., Leman, P.A., Swanepoel, R., 2005. An inhibition enzyme-linked immunosorbent assay for the detection of antibody to Rift Valley fever virus in humans, domestic and wild ruminants. J. Virol. Methods. 127, 10-18.

Paweska, J.T., van Vuren, P.J., Kemp, A., Buss, P., Bengis, R.G., Gakuya, F., Breiman, R.F., Njenga, M.K., Swanepoel, R., 2008. Recombinant nucleocapsid-based ELISA for detection of IgG antibody to Rift Valley fever virus in African buffalo. Vet. Microbiol. 127, 21-28.

R Development Core team, 2009. R: A language and environment for statistical computing. In: R foundation for statistical computing. ISBN 3-900051-07-0, Vienna, Austria.

Rimstad, E., East, N.E., Torten, M., Higgins, J., DeRock, E., Pedersen, N.C., 1993. Delayed seroconversion following naturally acquired caprine arthitis-encepahilitis virus infection in goats. Am. J. Vet. Res. 54, 1858-1862.

Roger, M., Girard, S., Faharoudine, A., Halifa, M., Bouloy, M., Cetre-Sossah, C., Cardinale, E., 2011. Rift valley fever in ruminants, Republic of Comoros, 2009. Emerg. Infect. Dis. 17, 1319-1320.

Sall, A.A., Macondo, E.A., Sène, O.K., Diagne, M., Sylla, R., Mondo, M., Girault, L., Marrama, L., Spiegel, A., Diallo, M., Bouloy, M., Mathiot, C., 2002. Use of reverse transcriptase PCR in early diagnosis of Rift Valley fever. Clin. Diag. Lab. Immunol. 9, 713-715.

Schmaljohn, C., Hooper, J.W., 2001. Bunyaviridae: the viruses and their replication, In: Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Straus S.E. (Eds.), Fields virology, fourth ed. Lippincott, Williams & Wilkins, Philadelphia, pp. 1581–1602.

Sissoko, D., Giry, C., Gabrie, P., Tarantola, A., Pettinelli, F., Collet, L., 2009. Rift Valley Fever, Mayotte, 2007-2008. Emerg. Infect. Dis. 15, 568-570.

Smithburn, K.C., Haddow, A.J., Gillett, J.D., 1948. Rift Valley fever; isolation of the virus from wild mosquitoes. Br. J. Exp. Pathol. 29, 107-121.

Suzich, J.A., Kakach, L.T., Collett, M.S., 1990. Expression strategy of a phlebovirus: biogenesis of proteins from the Rift Valley fever virus M segment. J. Virol. 64, 1549-1555.

Swanepoel, R., Coetzer, J.A.W., 2004. Rift Valley fever. In: Coetzer, J.A.W., Thomson, G.R., Tustin, R.C. (Eds.), Infectious diseases of livestock. Oxford University Press, Oxford, pp. 1037-1070.

Tortosa, P., Pascalis, H., Guernier, V., Cardinale, E., Le Corre, M., Goodman, S.M. Dellagi, K., 2012. Deciphering arboviral emergence within insular ecosystems. Infect. Genet. Evol. 12, 1333-1339.

Turell, M.J., Linthicum, K.J., Patrican, L.A., Davies, F.G., Kairo, A., Bailey, C.L., 2008. Vector competence of selected African mosquito (Diptera:Culicidae) species for Rift Valley fever virus. J. Med. Entomol. 45, 102-108.

Weidmann, M., Sanchez-Seco, M.P., Sall, A.A., Ly, P.O., Thiongane, Y., Lô, M.M. Schley, H., Hufert, F.T., 2008. Rapid detection of important human pathogenic Phleboviruses. J. Clin. Virol. 41, 138-142.

World Organisation for Animal Health, 2004. Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees), fifth ed. World Organisation for Animal Health, Paris, pp. 185-194.

Figures captions

Figure 1

(A) Alignment of nucleotides 2653 to 2850 of M segment from 52 strains from different origins. Identical nucleotides for all strains are represented by a dot. Strains are identified by their country of origin and date of isolation. Primers were represented on the consensus sequence of the alignment and designed from the most conserved parts of the alignment. Only the RVFV-M-2840R primer was degenerated for 2 nucleotides. (B) Nucleotide sequences, position on the M segment and annealing temperatures of the primers (Tm calculator http://www6.appliedbiosystems.com/support/techtools/calc/).

Figure 2

Comparative efficiency of the Taqman RT-PCR and the new RT-PCR of ten-fold serial dilutions of tissue culture infected supernatant starting from a Smithburn strain stock titrating to $10^{6.3}$ $TCID_{50}$/ml. Dilutions detected with (A) the Taqman RT-PCR (Bird et al., 2007) (B) the new RT-PCR .

Figure 3

Alignment of nucleotides 2653 to 2766 of M segment from 52 strains of different origin and from 30 strains detected with the new RT-PCR. Identical nucleotides for all strains are represented by a dot. Strains are identified by their country of origin and date of isolation. Anj: Anjouan, GC: Grande Comore, Mg: Madagscar, Mo: Moheli

Table(s)

Table 1
List of viruses tested with the newly developed RT-PCR detection system

Genus	Species	Vector-borne virus	RVF syndrome like associated	Main host
Phlebovirus	Belterra virus	*Culicidae*	-	Human
	Icoaraci virus	*Culicidae*	-	Human
	Punta Toro virus	*Culicidae*	-	Human
	Toscana virus	*Phlebotominae*	Fever	Human
	Sandfly Naples virus	*Phlebotominae*	-	Human
	Sandfly Sicilia virus	*Phlebotominae*	-	Human
Flavivirus	West Nile virus	*Culicidae*	-	Human/Animal
	Dengue type 1 virus	*Culicidae*	Fever	Human
	Dengue type 2 virus	*Culicidae*	Fever	Human
	Dengue type 3 virus	*Culicidae*	Fever	Human
	Dengue type 4 virus	*Culicidae*	Fever	Human
	Yellow fever virus	*Culicidae*	Fever	Human
	Japanese encephalitis virus	*Culicidae*	Fever	Human
	Apoï virus	NA	-	Human
Alphavirus	Chikungunya virus	*Culicidae*	Fever	Human
	O'Nyong nyong virus	*Culicidae*	Fever	Human
Orthobunyavirus	Schmallenberg virus	*Ceratopogonidae*	Abortion	Animal
Reovirus	Bluetongue virus serotype 2	*Ceratopogonidae*	Abortion	Animal
Morbillivirus	Peste des petits ruminants virus	NA	Abortion, Diarrhea	Animal
Influenza virus	H5N1 virus A	NA	Fever	Human/Animal

NA: Not Applicable

Table(s)

Table 2
Comparison of two RT-PCR detection systems on a collection of 2,756 serum samples from Madagascar and the Comoros archipelago (May 2010 to August 2011).

Collection area	Number of animals collected during the serosurvey	New RT-PCR, based on the M segment* (% of positive animals)	Taqman RT-PCR (Bird et al., 2007), based on the L segment (% of positive animals)	Incidence levels of seroconversions (%)
Mayotte	104	3/124 (0.29)	0/124 (0)	ND
Union of Comoros	182	20/1063 (10.9)	3/1063 (1.6)	14.4
Madagascar	204	15/1569 (7.35)	2/1569 (0.9)	4.94

*Each of the positive PCR sample detected with the new RT- PCR on M segment was confirmed by sequence analyses. ND: Not determined

Table(s)

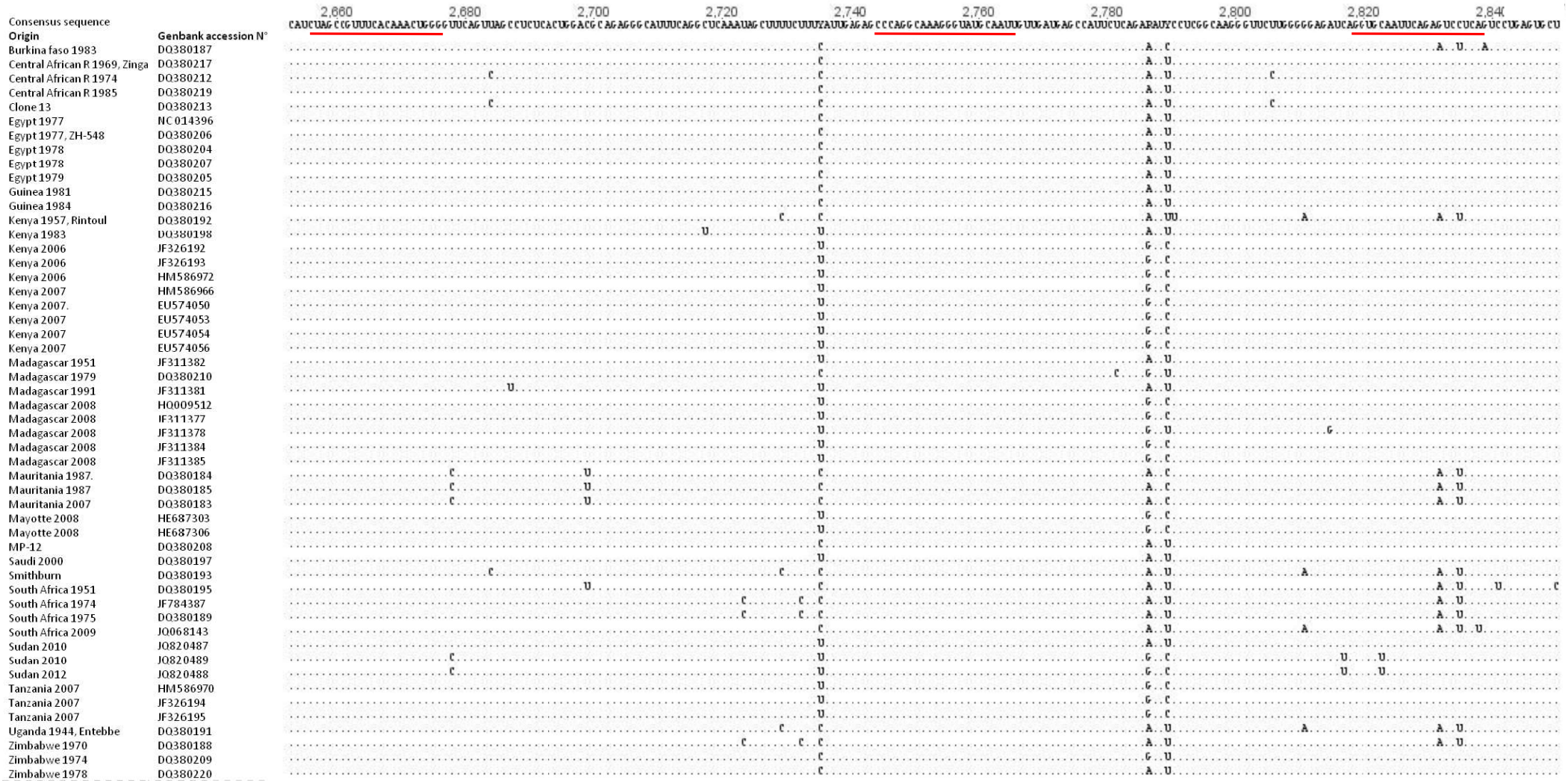
Table 3
Serological status of the 38 RT-PCR positive samples

Sample ID	Month 7 - 11/2010		Month 9 - 01/2011		Month 10 - 02/2011		Month 11 - 03/2011		Month 12 - 04/2011		Month 13 - 05/2011		Month 16 - 08/2011	
	Virology (new RT-PCR)	Serology (ELISA)	Virology (new RT-PCR)	Serology (ELISA)	Virology (new RT-PCR)	Serology (ELISA)	Virology (new RT-PCR)	Serology (ELISA)	Virology (new RT-PCR)	Serology (ELISA)	Virology (new RT-PCR)	Serology (ELISA)	Virology (new RT-PCR)	Serology (ELISA)
GCCMR0034	+	-	-	+	-	+	-	+	-	+	-	+	-	+
1ATV004	+	-	-	+	-	+	-	+	-	+	-	+	-	+
Mg 221 / 2EZM016	-	-	+	-	-	+	-	+	-	+	-	+	-	+
Mg 206 / 1AAR011b	-	-	+*	-	-	+	-	+	-	+	-	+	-	+
1AVO015	-	-	-	-	+	-	-	+	-	+	-	+	-	+
Mg 236 / 1ABE013b	-	-	-	-	+	-	-	-	-	+	-	+	-	+
1AJE018	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Mg 222 / 2ETa 023	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Mg 236 / 1ABE012	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Mg 264 / 1AAR009b	-	-	-	-	-	-	+	-	-	-	-	-	-	-
2ERA019b	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Mg 299 / 2FBE001	-	-	-	-	-	-	-	-	+	-	-	+	-	+
Mg 299 / 2FBE003	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Mg 275 / 2FFA030	-	-	-	-	-	-	-	-	+	-	-	-	-	-
GCCMR 0258	-	-	-	-	-	-	-	-	-	-	+	-	-	-
GCBMR 0246	-	-	-	-	-	-	-	-	-	-	+	-	-	-
ABMR 0029	-	-	-	-	-	-	-	-	-	-	+	-	-	-
ABMR 0030	-	-	-	-	-	-	-	-	-	-	+	-	-	-
CVII 28 / GCCMR1017	-	-	-	-	-	-	-	-	-	-	-	-	+	-
CVII 28 / GCCMR1020	-	-	-	-	-	-	-	-	-	-	-	-	+	-
CVII 31 / GCCMR1032	-	-	-	-	-	-	-	-	-	-	-	-	+*	-
CVII 40 / GCCSG0539	-	-	-	-	-	-	-	-	-	-	-	-	+*	-
CVII 46 / GCCSG0570	-	-	-	-	-	-	-	-	-	-	-	-	+	-
CVII 33 / GCBSG0507	-	-	-	-	-	-	-	-	-	-	-	-	+	-
CVII 39 / GCBSG0536	-	-	-	-	-	-	-	-	-	-	-	-	+	-
CVII 55 / GCBSG0615	-	-	-	-	-	-	-	-	-	-	-	-	+	-
CVII 66 / GCOSG0716	-	-	-	-	-	-	-	-	-	-	-	-	+	-
CVII 14	-	-	-	-	-	-	-	-	-	-	-	-	+	-
CVII 15 /ABMR0094	-	-	-	-	-	-	-	-	-	-	-	-	+	-
CVII 20 / ABMR0121	-	-	-	-	-	-	-	-	-	-	-	-	+	-
ABMR 0040	-	-	-	-	-	-	-	-	-	-	-	-	+	-
CVII 3 / MOCMR0071	-	-	-	-	-	-	-	-	-	-	-	-	+	-
CVII 3 / MOCMR0072	-	-	-	-	-	-	-	-	-	-	-	-	+	-
2DMA029	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Mg314 / 2DNJ023	-	-	-	-	-	-	-	-	-	-	-	-	+*	-
SG 134	-	-	-	-	-	-	-	-	-	-	-	-	+	-
MJ 15	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Abortion	-	-	-	-	-	-	-	-	-	-	-	-	+*	-

*Samples detected by the Taqman RT-PCR (Bird et al., 2007)

Figure(s)

A.



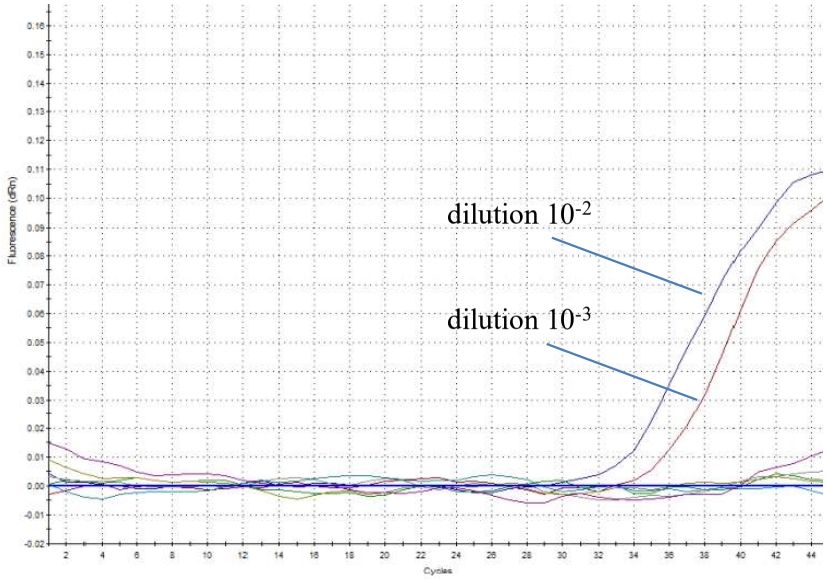
B.

Primer	Sequence	Nucleotide position on the M segment	Annealing T
RVFV-M-2656F	5′-CTAGCCGTTTCACAAACTGGG-3′	2,656 - 2,676	60
RVFV-M-2840R	5′-GACTGARGAYTCTGAATTGCACC-3′	2,817 - 2,840	55
RVFV-M-2766R	5’-CAATTGCATACCCTTTGCCTGGGC-3’	2,742 - 2,766	70

Figure 1

Figure(s)

A.



B.

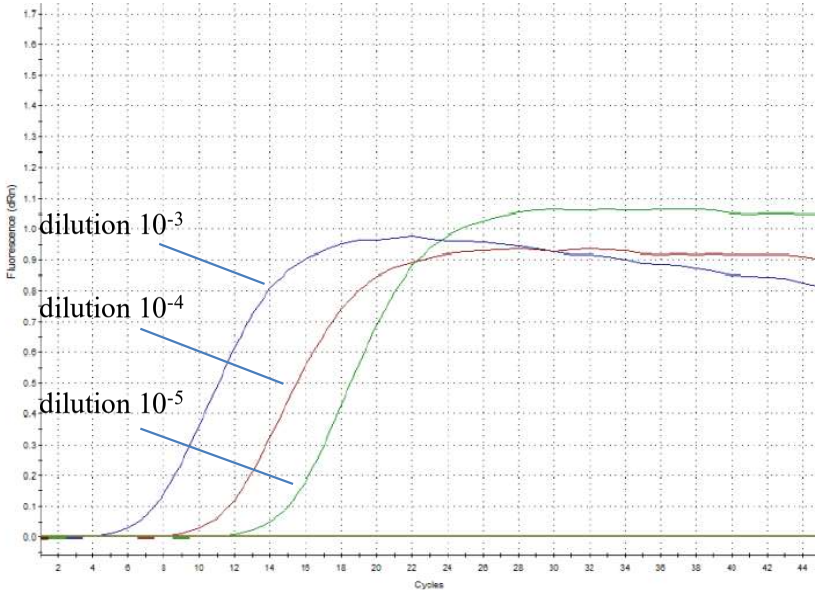


Figure 2

Figure(s)

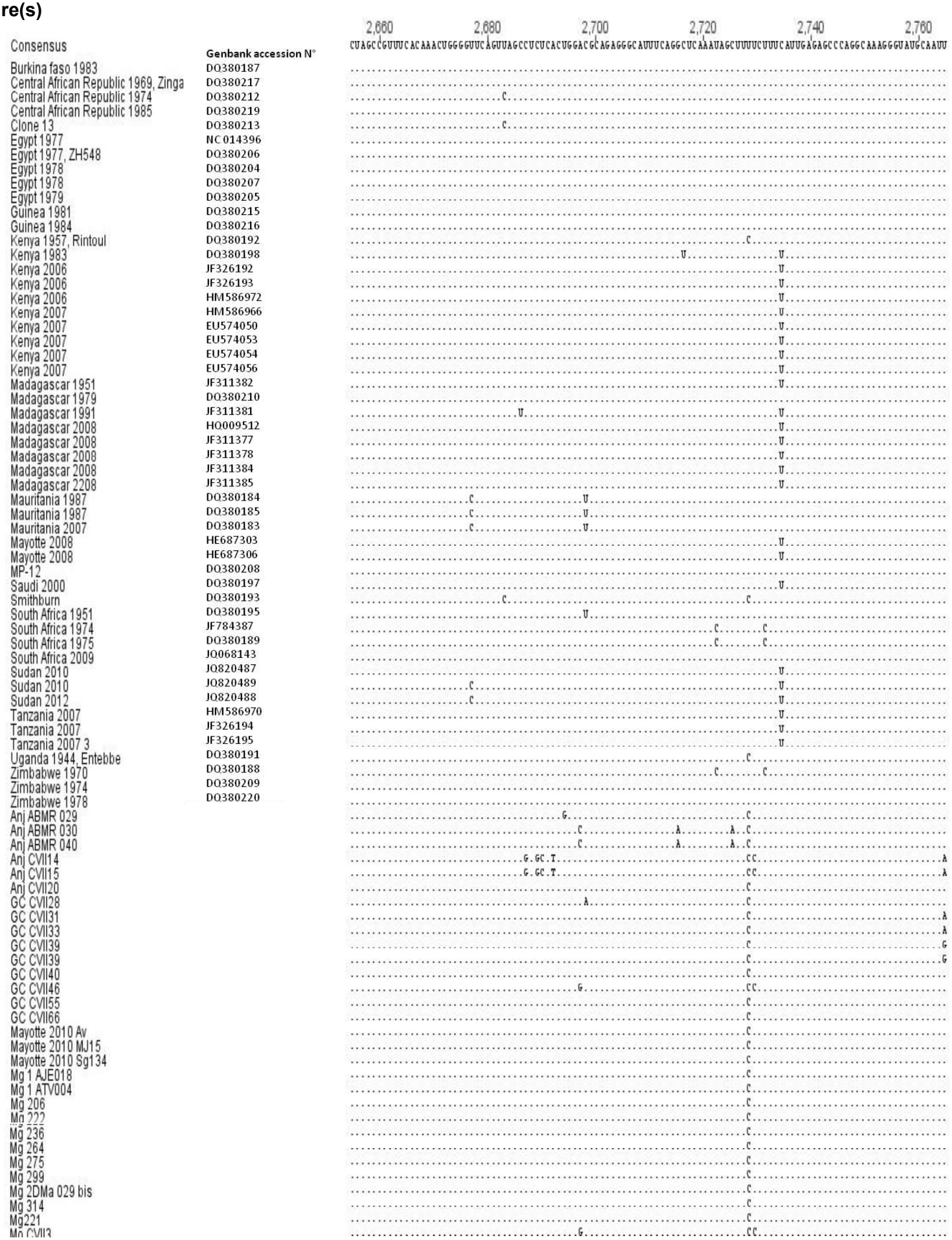


Fig.3.

Highlights

- A highly sensitive real-time PCR assay for the detection of Rift Valley Fever virus
- Sensibility tested on 2,756 serum from Madagascar and the Comoros archipelago
- Comparison with a RVF specific quantitative real time RT-PCR reference technique
- System specific and 7.7 times more sensitive than the reference technique
- Diagnostic tool for the detection of low viral loads in inter-epizootic periods