

Development of a diagnostic one-tube RT-PCR for the detection of Rift Valley fever virus

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ABSTRACT

ESPACH, A., ROMITO, M., NEL, L.H. & VILJOEN, G.J. 2002. Development of a diagnostic one-tube RT-PCR for the detection of Rift Valley fever virus. *Onderstepoort Journal of Veterinary Research*, 69:247–252

Diagnosis of Rift Valley fever (RVF) is based on serology and virus isolation. The disadvantages of the former include poor sensitivity, high cost, risks associated with using infectious virus as antigen, the lengthy duration of ELISA as well as cross-reactivity with other *Phleboviruses*. We developed, optimised and evaluated a one-tube reverse-transcription-polymerase chain reaction (RT-PCR) for the detection of Rift Valley fever virus (RVFV) in ruminants. The PCR primers for this assay were designed to anneal to a region within the M segment of the virus genome, encoding glycoproteins G1 and G2. A PCR amplicon of 363 bp was obtained. The sensitivity of the assay was determined to be 0.25 TCID₅₀. This test should allow for the early and rapid detection of RVFV in both serum and whole blood. In addition, it could facilitate the quantification of antigen for the manufacture of current vaccines.

Keywords: Diagnosis, M segment, one-tube RT-PCR, Rift Valley fever

INTRODUCTION

Rift Valley fever (RVF) was first described in 1931 by Daubney, Hudson & Garnham as a usually mild to inapparent mosquito-borne disease. It may, however, manifest as an acute or peracute disease of domestic ruminants (sheep, goats and cattle) especially in Africa. Pregnant and neonatal animals are particularly susceptible and it can also be a potentially serious zoonosis. Recent outbreaks outside endemic areas have emphasised the importance of

an efficient surveillance programme. The aetiological agent, Rift Valley fever virus (RVFV), has a single-stranded, negative-sense RNA genome (Shope, Peters & Walker 1980; Levy, Fraenkel-Conrat & Owens 1994) and is a member of the *Phlebovirus* genus in the *Bunyaviridae* family. The genome consists of three single-stranded RNA segments, designated S, M and L according to the size of the segments (Robeson, El Said, Brandt, Dalrymple & Bishop 1979; Bishop, Calisher, Casals, Chumakov, Gaidamovich, Hannoun, Lvov, Marshall, Oker-blom, Petterson, Porterfield, Russel, Shope & Wetaway 1980; Levy *et al.* 1994). Each segment is enclosed within a separate nucleocapsid. The M segment codes for the structural glycoproteins G1 and G2 (55–70 kDa), located on the viral envelope, as well as two non-structural proteins (14 kDa and 78 kDa) (Gentsch & Bishop 1979; Suzich, Kakach & Collet 1990). Both G1 and G2 have been shown to induce neutralising antibodies (Gentsch *et al.* 1979).

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Laboratory diagnosis of RVF is based on histopathology, serology (including complement fixation, ELISA, haemagglutination inhibition and virus neutralisation), virus isolation and/or antigen detection (including immunofluorescence, immunoperoxidase staining and ELISA) in serum or tissue samples collected during the febrile stage of disease (Barnard 1996). Virus isolation is time consuming and is a health risk to laboratory workers. Diagnosis on the basis of serology may be complicated by unwanted cross-reactivity (Swanepoel, Struthers, Erasmus, Shepherd, McGillivray, Shepherd, Hummitsch, Erasmus & Barnard 1986). Such assays nevertheless have the advantage that only inactivated antigen is used (Barnard 1996; Swanepoel *et al.* 1986). The currently used ELISA can distinguish between early (IgM-based) and late (IgG-based) current infection, and disease acquired previously (Niklasson, Peters, Grandien & Wood 1984). Whole virus is used as antigen and although it is inactivated, the initial handling of infectious virus constitutes a health risk. Furthermore, the ELISA lacks sensitivity and is time-consuming and costly. Recently, a RVFV specific RT-PCR has been described (Sall, Thonnon, Sene, Fall, Ndiaye, Baudez, Mathiot & Bouloy 2001). This assay is a one-tube two-step nested RT-PCR. Primers were designed to bind to a region on the S segment of the viral genome, which encodes the non-structural NS proteins of the virus. We too have developed a RVFV-specific diagnostic RT-PCR to improve diagnostic sensitivity, safety and speed, and which can also be used as a tool to quantify virus during the process of classical vaccine production. In our case we targeted the M segment of the RVFV genome. We here describe the development, optimisation, evaluation and validation of a one-tube, non-nested RT-PCR for the early detection of RVFV RNA in serum or blood samples.

MATERIALS AND METHODS

Primers

Primers ARV1-F (forward) and ARV2-R (reverse) were designed to anneal at positions 1755–1775 and 2097–2117 respectively on the M segment of the viral genome. The resulting 363 bp region encompasses the 3' end of the G2 gene and the 5' end of the G1 gene. Both primers were synthesised by Gibco BRL (UK). The sequences were:

ARV1-F: 5'-TGTCACACTGCTCTCAGTGCC-3'

ARV2-R: 5'-GGAGCTTGCCTGAATCTGTTG-3'.

PCR optimisation

Optimisation was performed using plasmids pSCRV-6 and pCI-G2G1-EGFP. Each plasmid incorporates a region of the M segment containing the two envelope glycoprotein genes. Initial titrations of MgCl₂ concentrations were performed for RT-PCR using standard procedures (Sambrook, Fritsch & Maniatis 1989; Boehringer Mannheim 1995). The optimal reaction conditions were: 5 pmoles of each primer in a 25 µl reaction volume together with 5 U of Ex Taq (Takara); 2.5 µl of 10x buffer containing 25 mM MgCl₂; 10 mM dNTP's and 3 ng of template plasmid DNA. The time-temperature profile was 94 °C for 30 s; 61 °C for 30 s; 72 °C for 30 s for 30 cycles.

RNA isolation and reverse transcription

The Smithburn strain of RVFV was used as template for further optimisation. Different RNA extraction methods were evaluated, including that of Chomzynski & Sacchi (1987) and a commercially available kit. Naïve blood and serum was spiked with 100 TCID₅₀ of the virus stock in either 140 µl (commercial viral RNA extraction kit method) or 200 µl of the extraction volume (Chomzynski & Sacchi 1987). This corresponded to a titre of 7x10² TCID₅₀/ml and 5x10² TCID₅₀/ml, respectively. Only the extraction method described by Chomzynski and Sacchi (1987) was used to extract RNA from the serum samples. A one-tube RT-PCR was then performed following RNA extraction. The reverse transcription step was performed at 37 °C for 30 min and the PCR was done as follows: 94 °C, 30 s; 65 °C, 30 s; 72 °C, 30 s for 35 cycles. The total reaction volume (25 µl) contained 5 pmoles of each primer, 20 U MMLV-RT (Promega), 1.25 U Ex Taq (Promega), 10x supplied PCR buffer (20 mM MgCl₂), 10mM dNTP's, 1U HPRI and 5 µl extracted RNA template.

Sensitivity assays

Sensitivity was determined as follows: 1µl of the virus stock (10⁶ TCID₅₀/ml) was added to 100 µl phosphate buffered saline (PBS) to which an equal volume of naïve serum or blood was added (i.e. 10³ TCID₅₀ or an initial dilution of 10⁻³). A ten-fold dilution series was then performed, yielding 1 TCID₅₀ at the final dilution of 10⁻⁶. RNA extraction was performed according to Chomzynski & Sacchi (1987) on each of the 200 µl samples. One-tube RT-PCR was then done as described using 5 µl of the 20 µl extracted RNA sample.

PCR validation

Eighteen serum samples were obtained from vaccinated and experimentally infected sheep during a RVF animal vaccine trial at the Onderstepoort Veterinary Institute (OVI) in collaboration with Onderstepoort Biological Products (OBP). In addition, several serum samples found to be positive by ELISA, were obtained from the Senegalese Institute for Agricultural Research, Dakar, Senegal. These and other serum samples were stored at -70°C before and after use with a minimum of freeze-thawing cycles. RNA was extracted and used as template.

RESULTS

Primers and PCR optimisation

A primer-pair specific for a region within the M segment was designed from published RVFV sequence data (Collett, Purchio, Keegan, Frazier, Hays, Anderson, Parker, Schmaljohn, Schmidt & Dalrymple 1985). During the process of optimisation, a 363 bp amplicon was generated following RT-PCR, using as template plasmid DNA containing the glycoprotein-encoding region from the M segment of the virus genome (G2G1).

RNA isolation and reverse transcription

Blood and serum was spiked with 50 μl of a 10^6 TCID₅₀/ml virus stock (5×10^4 TCID₅₀). The relative intensity the amplicon bands (Fig. 1, lanes 4 and 5) after agarose gel electrophoresis suggested that the RNA extraction method described by Chomzynski & Sacchi (1987) was superior to the commercial viral RNA extraction kit (Fig. 1, lanes 7 and 8). This was found for both blood and serum samples (not shown for the latter). This extraction procedure is also less expensive than the commercial one. The optimal MgCl₂ concentration was found to be 2.5 mM.

Sensitivity assays

Serial dilutions using a 10^6 TCID₅₀/ml virus stock were prepared in serum and blood as described under Methods. The RT-PCR was performed after extraction of the RNA and amplicons detected following 1.5% agarose gel electrophoresis (Fig. 2). The highest dilution from which a 363 bp amplicon could still be obtained from serum was 10^{-6} (Fig. 2, lane 4), equivalent to 1 TCID₅₀. Since only one-quarter of the RNA extracted from this sample was used for the RT-PCR assay, the sensitivity was cal-

culated to be 0.25 TCID₅₀. This is in agreement with that obtained from similar assays for the detection of other RNA viruses (Bremer & Viljoen 1998; Sall *et al.* 2001).

PCR validation

The samples used for validation were collected from sheep participating in a RVF vaccine trial at the OVI. These sheep were immunised with a DNA vaccine and challenged with a virulent RVFV isolate. All animals as well as non-immunised ones used in the above experiment, tested positive after challenge using the RT-PCR diagnostic assay (Fig. 3). The presence of replicating virus in immunised sheep suggests that sub-optimal protection was induced after DNA vaccination. The amplicons obtained in the positive vaccine control animals (immunised with a conventional inactivated vaccine) confirmed the efficacy of the RT-PCR in detecting RVFV *in vivo*, even though only limited wild-type viral replication post challenge was expected to have taken place. Naïve serum was used as the

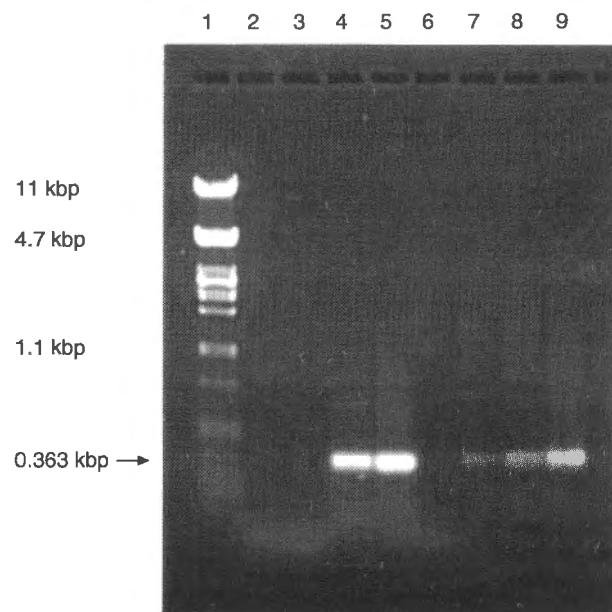


FIG. 1. Agarose gel indicating the 0.363 kbp amplicons generated from templates extracted by two different methods. These were the one-step procedure described by Chomzynski & Sacchi (1987) (lanes 3–5) and a commercial viral RNA extraction kit (lanes 6–9).

10^6 TCID₅₀/ml RVF virus stock (2×10^{-5} TCID₅₀) was used to spike blood. Molecular mass marker, λ DNA digested with Pst I (lane 1); water control (lane 2); naïve blood using the one-step procedure (lane 3); spiked blood (lane 4); cell culture supernatant containing virus (lane 5); naïve blood (lane 6); spiked blood (lane 7); virus stock (lane 8); plasmid control (lane 9).

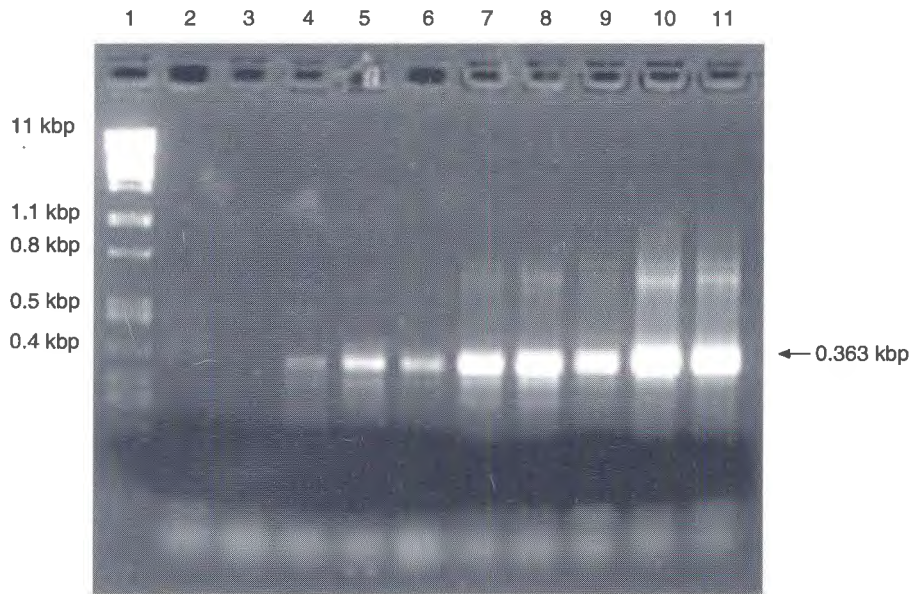


FIG. 2 Virus stock was serially diluted in test serum and the RNA was extracted to determine the relative sensitivity of the one-tube RT-PCR. Molecular mass marker, λ DNA digested with Pst I (lane 1); water control, naïve serum and dilutions of 10^{-6} , 10^{-5} , 10^{-4} , 10^{-3} , 10^{-2} , 10^{-1} , respectively (lanes 2–9); serum spiked with $6.25 \mu\ell$ of the of 10^6 TCID/ml virus stock (lane 10), $200 \mu\ell$ of 10^6 TCID/ml virus stock (2×10^5 TCID₅₀) (lane 11). The weakest visible band was obtained from the final dilution of 10^{-6}

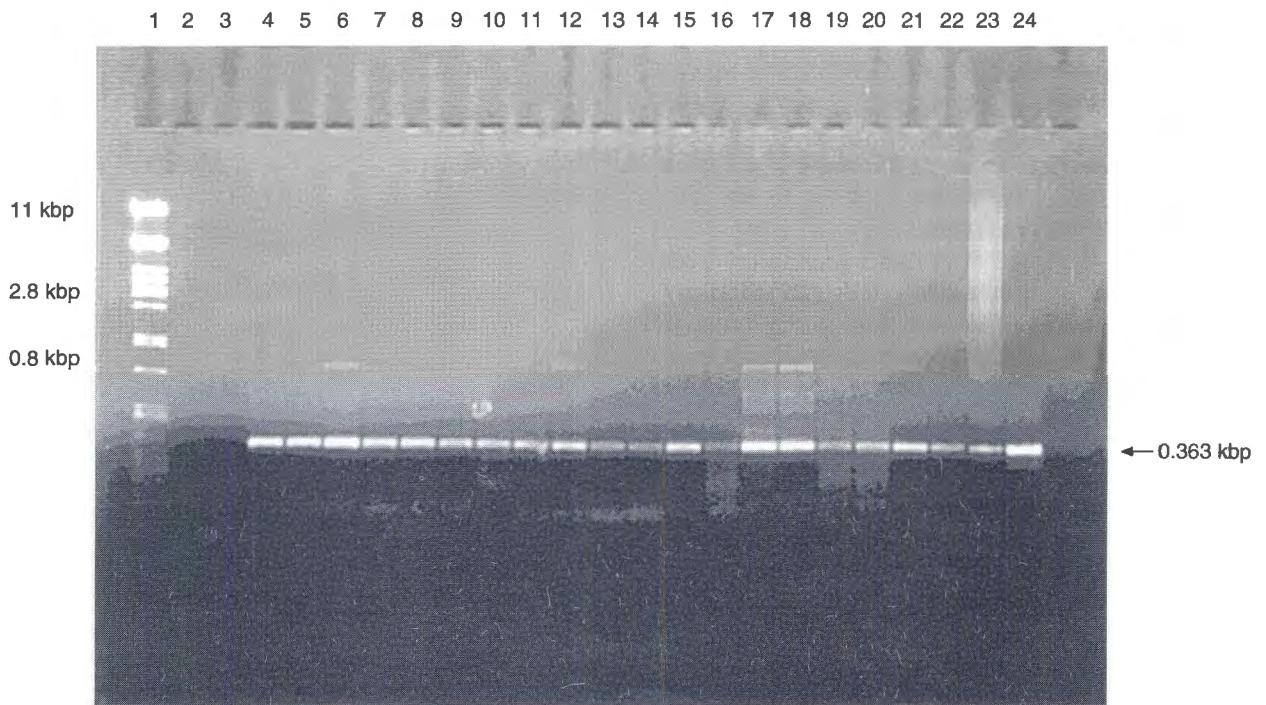


FIG. 3 RT-PCR amplicons derived from sera of animals used in a vaccine trial as described. Extraction of viral RNA was performed using the method described by Chomczynski & Sacchi (1987). λ DNA digested with Pst I (lane 1); water control (lane 2); naïve serum control (lane 3); sera of sheep after challenge with a virulent RVFV isolate (lanes 4–24)

negative control and no amplification was evident (Fig. 3, lane 3).

Sera of field samples obtained from Senegal were not only shown to be positive in an ELISA for the presence of RVFV-specific antibodies, but also for the presence of viral RNA using RT-PCR (data not shown).

DISCUSSION

RVF is a potentially serious human and veterinary disease. An outbreak can have important economic implications due to death and abortions in neonatal and pregnant animals. The recent outbreaks in Saudi Arabia and Yemen emphasise the importance of preventing the spread of the virus to non-endemic areas. Rapid diagnosis and an efficient surveillance programme are crucial to effective control. Whole virus is used as antigen in currently employed diagnostic assays. These assays such as ELISA, HI, VN, are laborious and time-consuming. It is not always possible to detect virus using these procedures, and antibody titres might also be too low for adequate detection. The need to handle virus for use as antigen for serological assays also presents a health risk to laboratory personnel. The replacement of these assays with a one-step RT-PCR assay would allow for a safer, more accurate and timely diagnosis of the disease.

We designed specific primers for the purpose of developing such an assay. Two RNA extraction methods were examined and the method described by Chomczynski & Sacchi (1987) was shown to be preferable. Serum was also found to be the better source of virus-specific RNA. The optimal $MgCl_2$ concentration for the PCR reaction mixture was shown to be 2.5 mM. Sensitivity of the one-step RT-PCR was determined to be 0.25 TCID₅₀ in sera inoculated with virus-containing cell-culture supernatant. It is expected that the ability of the assay to detect non-viable virus particles would contribute to its superior sensitivity over conventional virus isolation methods. The sensitivity obtained was similar to the sensitivity of RT-PCR for detecting other RNA viruses (Bremer & Viljoen 1998; Sall *et al.* 2001). Further evaluation of the assay was done using different serum samples from sheep immunised with a DNA vaccine containing the RVFV glycoprotein gene. After a boost, the sheep were challenged with a virulent virus isolate obtained from an African buffalo (*Syncerus caffer*) infected during an outbreak in the Kruger National Park in South Africa. In

addition, sheep sera obtained from the Senegalese Institute for Agricultural Research were also tested, initially by ELISA and then by RT-PCR. The latter confirmed the presence of RVFV RNA in both sets of sera. In the case of our trail sheep it is indicative of virus replication in DNA immunised sheep suggestive of sub-optimal protective immunity. The intensity of the bands was found to vary and this could be an indication of variable levels of replicating virus within the sheep.

This one-tube non-nested RT-PCR is to our knowledge the first described which targets the glycoprotein genes encoded by the M segment of the virus genome. In comparison to the RT-PCR assay described by Sall *et al.* (2001) which targets the coding region of the NS protein on the S segment of the virus genome, our assay appears to be more sensitive. The former nested RT-PCR employed by Sall *et al.* (2001) gave a sensitivity of 0.5 pfu, while a one-step method without a nested step they employed had a sensitivity of only 50 pfu. Our non-nested one-step RT-PCR had a sensitivity of 0.25 TCID₅₀. This is equivalent to 0.17 pfu (TCID₅₀ × 0.69 = pfu) (O'Reilly, Miller & Luckow 1992).

In addition to the rapid diagnosis of RVF in infected animals, quantification of the viral load as required for classical vaccine production is also possible. RT-PCR is also a safer and more rapid method for detecting viral antigen than virus isolation procedures, since handling of virus as antigen source is obviated. Cytopathic effects may become detectable after one to five days after inoculation of cell cultures during virus isolation but antigen detection can be accelerated by means of performing IF on the infected cultures (Swanepoel & Coetzer 1994). This can be done 24h after infection of the cells. A RT-PCR can be performed on the sample of question immediately after receiving it with results being available within three to four hours. Antibody levels can be detected only from Day 3 after infection (Swanepoel *et al.* 1986) while RT-PCR can potentially detect the virus at an earlier stage, enabling a more rapid diagnosis after infection than is possible with methods such as PRNT₈₀ (Swanepoel & Coetzer 1994).

ACKNOWLEDGEMENTS

We thank Dr Yaya Thiongane of the Senegalese Veterinary Institute, Dakar, Senegal for providing us with sheep serum samples.

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