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Preparation and Optimization of Rift Valley Fever Antigen and Sheep Anti-RVF Antibody for Sero-Diagnosis of Rift Valley Fever

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Abstract

Rift Valley fever (RVF) is a zoonotic, arthropod–borne RNA viral disease that may cause abortions in pregnant ruminants and haemorrhage in humans. RVF vaccination is currently unstable and short-lasting warranting need for safe, specific and sensitive diagnostic tools for veterinary and surveillance laboratories. The objectives of the study were to culture RVF virus (antigen) *in vitro* using African monkey kidney (Vero) cells; to identify the extracted antigen using Immunofluoresce antibody (IFA) test and to optimize the antigen and RVF-positive serum sample (antibody) obtained from sheep (sample H2550) using Indirect ELISA for designing an ELISA-based diagnostic kit. The experimental study was done at KARI- Biotechnology Centre between August 2008 and December 2008. RVF virus was cultured and sub-cultured *in vitro* using Vero cells and identified. Indirect ELISA, modelled as a titration chequerboard, was done using the extracted antigen and antibody. The readings from the ELISA were done at an optical density (OD) of 405nm. An OD of approximately 1 was sought, this being a representation of the optimum concentrations of antigen and positive serum. The standardization study revealed the optimum concentrations of the RVF antigen and positive serum (Sheep H2550) to be 1:80 and 1:27, respectively. Validation of optimized antibody and antigen concentrations using a wide range of samples from different species and localities was recommended.

Keywords: Antigen; Indirect ELISA; Rift Valley Fever; Sheep anti-RVF antibody

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Introduction

Rift Valley Fever (RVF) is an arthropod-borne viral disease of importance in public health because it causes epizootics of abortion and high mortality in domestic animals during which humans become infected (Meegan and Bailey, 1989; Gonzales-Scarano and Nathanson, 1996). The Rift Valley Fever virus that causes the zoonotic haemorrhagic viral belongs to Bunyaviridae family and genus Phlebovirus and is 3-stranded RNA virus that is transmitted by a mosquito vector (Vialat et al., 1997). The RVF outbreak that occurred in Kenya during 2006/2007 killed about 75 people; hence the need to develop RVF diagnostic kits that are validated to African settings and more so, development of safe and stable vaccines for livestock and humans. The use of locally obtained samples that are sero-positive and negative for RVF as well as use of locally grown RVF antigen in cell cultures enables the cost effective development of an ELISA diagnostic kit that can be used to test other sera samples. This project aimed at producing optimised RVF antibody and antigen that would be used in a diagnostic kit of ELISA. The virus is cytopathic and forms plaques (Gonzales- Scarano and Nathanson, 1996). The plaques thus characterise *in vitro* infection of the cell culture with RVF but verification is made using confirmatory tests like Immunofluoresce antibody (IFA) test or ELISA. RVF antigen extraction may be by suspension in borate buffer or saturated ammonium sulphate. Monkey kidney (Vero) cells grown *in vitro* are inoculated with the virus for subsequent extraction of the viral antigen. Sero-diagnosis widely utilises immunoassays (Ngichabe and Soi, 1999). Selected for detection of RVF virus antigen was the Immunoflouresce antibody (IFA) test which is not only readily available, but is arguable the most rapid and precise method for identification of RVF, considering that monoclonal antibodies for RVF are used and that it and can be used in conjunction with cell culture. For optimisation of RVF virus antigen, Indirect ELISA provides highly specific, accurate and readily available method (Zaki *et al.*, 2006), and was thus used. The objectives of the study were to culture RVF virus (antigen) *in vitro* using African monkey kidney (Vero) cells; to identify the extracted antigen using Immunofluoresce antibody (IFA) test and to optimize the antigen and RVF-positive serum sample (antibody) obtained from sheep (sample H2550) using Indirect ELISA for designing an ELISA-based diagnostic kit.

Materials and Methods

Experimental Design

The experimental project utilised materials and reagents for culturing RVFV *In vitro*, extracting and purifying the RVF antigen as well as optimising it together with RVF-positive sera from sheep using Indirect ELISA. The procedures and activities followed to achieve this constitute the design of the study. The experimental study was done at KARI-Biotechnology Centre between August 2008 and December 2008.

Standard Operating Procedures used:

Preliminary activities: The ultra violet light in the Biosafety hood was turned on 10min prior to working in the hood, and 10min after working with infectious material. Whilst working in it, the air blower was kept on. To avoid contamination while working in the hood, hands and all materials that enter the hood were first sterilized by swabbing well in 70% alcohol. While in the hood, lids and bottle/flask necks were exposed lightly to the flame before handling media, Trypsin or cell culture solutions. Needed quantities of media and Trypsin

were first aliquoted from their stock solutions before pipetting after which the used pipettes were discarded. Prior to working in the hood all equipment and surfaces in the hood were sterilized with 70% alcohol.

This also applied to surfaces on to which TC flasks were placed. During aliquoting of solutions or any work in the hood, handling of bottles/flask necks and interior of lids was avoided.

Preparation of Media and Trypsin solution: ***(a) RPMI-1640 Media:*** This was the medium used for *in vitro* culturing of monkey kidney (Vero) cells. It was the source of nutrients for the growing cells and contained Foetal Bovine Serum (FBS) for cushioning cells, provision of growth factors and protecting cells from *in vitro* shock; L- Glutamate, an essential amino acid for cell growth; Antibiotic, PenStrep and Antifungal agent, Amphotericin to protect cell cultures from contamination. Its preparation was done thus:***(i)*** Sterilisation of 450ml RPMI- 1640 base (with L-Glutamate) using a 0.2 µm filter in the laminar flow chamber. ***(ii)*** Aseptic addition of 10ml PenStrep and 10 ml Amphotericin at 37ºC into the sterile RPMI-1640 base.***(iii)*** Aseptic addition of 50ml FBS at 37ºC into the sterile RPMI solution to make RPMI-1640 media.***(iv)*** The media was always kept at 4ºC when not in use. ***(b) Trypsin Solution:*** Trypsin solution was used to split (passage) Vero cells by detaching the adherent cells and lysing cell- cell bonds. Its preparation was done thus:***(i)*** 40ml of 10x solution A (containing 80g NaCl, 4g KCl, 3.5g $NaHCO_3$, 10g Glucose, 100ml of 0.1% Phenol Red topped up to 1 litre with dH_2O) and placed in a 500ml beaker. ***(ii)*** 5ml 1% Versene and 2 ml of 5% $NaCO_3$ added and the solution topped up to 500ml with dH_2O. ***(iii)*** The Trypsin solution was then sterilised using a filtration unit, and stored at 4ºC always when not in use.

Culturing of Vero Cells: This is the multiplication or bulking of Vero cells to obtain large quantities that can carry and multiply the RVF virus. Culture media was obtained from -4ºC and placed in a 37ºC water bath for 15mins. Addition of media and healthy Vero cells into T25 flasks was done aseptically in the Biosafety hood. The media was aliquoted into sterile 50ml falcon tube(s) and a sterile 10ml pipette used to obtain media from the tube and into the T25 flask. Only one pipette was used per flask. The flasks containing cell cultures were labelled with User Initials, Date, Type of cells (Vero) and Cell line after which they were transferred to the Incubator (with CO_2 supply and 37ºC). In the incubator, the flask lids were slightly loosed to allow CO_2 flow. Cultures were monitored daily to check for growth rate, presence of contamination or depletion of nutrients. After 3 days, a confluent monolayer was often observed under an Inverted microscope. Cultures that showed bacterial or fungal contamination were discarded. On attaining a confluent monolayer and overgrowth of cells (seen as rounded cells in suspension), splitting was done. This was usually after the 3rd day of the initial culture.

Sub-culturing of Vero cells**:** This is a splitting or passaging process useful in cell multiplication and provision of new nourishment to the growing cells, and it was done using Trypsin solution. The solution was obtained from 4ºC refrigeration and placed in a 37ºC water bath for 15 mins. The T25 flasks with cells to be passaged had their lids and necks sterilised by exposure to dry heat in the hood. The culture suspension was discarded, 4 ml Trypsin added aseptically to each flask, and the flasks swirled for about 5 seconds. This Trypsin is consumed by the serum, FBS. The flasks were added with a further 2ml Trypsin

which passages the cells. Observation was then done under microscope to check the extent of detachment. The flasks were tapped gently on the side to facilitate cell detachment so that cells appear singly. Pressure-pipetting was then done in the hood to break cell clumps.

Half the cells were then transferred into an empty sterile T25 flask and media added to both parent and daughter flasks so that each flask contained 8ml culture solution. The sub-culturing process was done quickly since prolonged exposure of Trypsin to the cells lyses their membranes; also, prolonged exposure to light degrades cells. The culturing process continued, with passaging done after a confluent monolayer was achieved (often within 3-4 days) and media changed the day after passaging. Cultures in T25 flasks were passaged into T75 and T75 monolayer cell cultures split into T175 flasks. The volume of Trypsin and media used to passage and grow cells is elucidated in Table 1. This process was systematically sustained until a total of 8-T175 flasks with confluent culture monolayer were obtained. More RPMI-1640 media and Trypsin solutions were prepared well before being depleted, and their sterility was tested by placing aseptically a few millilitres into one clean T25 cell culture flask under incubation, and observing the test TC flask using the inverted light microscope for any contamination after 24 hours. Sterility test for the RVF inoculum was done in the same way.

Inoculation of RVFV: Safety was observed when handling RVF inoculum. Gloves were worn, sterilisation of equipment done and infected materials to be discarded placed in safety bags for autoclaving prior to incineration. The RVF virus was retrieved from storage at -20ºC and placed in 37ºC water bath to thaw. On passing the sterility test, inoculation was done to 3-4 day old Vero cells having a confluent monolayer. A live attenuated RVF virus constituted the inoculum. It was obtained from National Veterinary Laboratories, Kabete and had been developed by serial-culturing it *in vitro* until it lost virulence. 1ml of the sterile inoculum was used per T175 flask. Media from 6-T175 flasks was discarded and the inoculum added. These flasks were incubated for 30 minutes to allow the RVF virus to adsorb. 10ml of media was then added to these flasks to sustain the cells. 1- T175 flask acted as a negative control and had only 10ml media changed to it. The flasks were then placed in the incubator. 1 T25 flask with a confluent young monolayer was also inoculated with 1 ml of virus and another ml of media added after letting the virus to adsorb. This T25 culture would be used to confirm presence of RVF virus. Regular observation was done for all flasks under the light microscope. Cytopathic effect (CPE) was expected in 3-4 days. 10- 20% CPE was targeted so that harvesting of the cells with RVF would be done.

***Confirmation of Virus*: **The infected T25 culture showing CPE similar to the other T175 cultures was obtained and used to confirm presence of the RVF virus using the Immunofluoresce antibody technique, so that if found positive for the RVF virus, a conclusion would be made that the cultured Vero cells were RVF-infected and competent for further work. Infected Vero cells in the T25 flask were harvested by scrapping them off using a cell scrapper. This was performed in the hood and under strict observations to safety and sterility. The cell suspension was aseptically placed in a 15ml falcon tube for centrifugation at 2500rpm for 15min. The supernatant was discarded and the pellet reconstituted in

some back flow supernate. 100µ l of this pellet was picked and 2 drops spotted on 2 separate wells of a clean slide for Immunofluoresce antibody (IFA) test. 2 drops of a negative control (derived from clean Vero cell culture) were spotted on 2 separate wells as were a further 2 drops of positive control (from RVF confirmed cultures). The respective test and control wells were labelled in pencil. The slide was left to air-dry then immersed in a jar of acetone to fix the cells.

After 15mins, the slide was retrieved and left for a while to air-dry. A drop of RVF monoclonal antibody was added to each well after which the slide was placed in a moist chamber and incubated at 37ºC for 30mins. This slide was washed once in PBS and left to air-dry. A drop of isothiocyanate conjugate (a fluorescent-labelled secondary anti-RVF antibody) was added to each well and incubation done at 37ºC in a moist chamber for 30 minutes. The slide was then washed once in PBS and air dried. Observation was made under oil immersion using 100x U.V microscope. Green fluorescence (emanating from the cytoplasmic and nuclear regions of the cells) was expected from the wells coated with cells infected with RVF virus and none from the negative control. This is a confirmatory test of the presence of RVF Antigen in the infected Vero cells.

Purification of Antigen (RVF virus): 6-T175 Vero cell cultures, cultured normally and infected with a live attenuated RVF inoculum, were harvested aseptically using cell scrapers. The viral suspension was placed in 3-50 ml falcon tubes and spun at 4000rpm for 20 mins to collect the pellet which are cells with virus. Supernatant in each falcon tube was discarded to leave a pellet which was washed thrice with borate buffer saline, then spun at 4000rpm for 10min to suspend the impure supernate which was discarded. The pellet was constituted in 4ml of the borate saline. [Borate buffer at pH 9 was made by mixing 3.1g of Boric acid and 4.763g of Sodium tetraborate in 250ml of distilled water]**.** The cells in the buffer were sonicated for 20secs using the Branson probe sonicator at setting 4, so as to lyse the cells and release the RVF virus. The suspension was then centrifuged at 4000rpm for 30mins to collect a pellet of cell debris. The suspension was thus collected as the RVF viral antigen.

Optimisation of RVF Antigen Using Indirect Elisa**:** The plate layout of ELISA titration chequerboard is shown as Figure 1. ***Indirect ELISA Test Procedure:*** Preparation of reagents and working dilutions was carried out thus:**(i)** PBS, 0.01M, pH 7.4. 1 sachet dissolved per litre of dH_2O.**(ii)** Washed buffer; 5ml Tween 20 diluted with PBS to a final concentration of 500ml**. (iii)** Diluent buffer; 2% skim milk in PBS or 2g skim milk in 100ml PBS**. (iv)** Blocking buffer; 10% skim milk in PBS or 5g skim milk in 50ml PBS. Coating buffer; at pH 9.6, made by mixing 1.59g sodium carbonate and 2.93g sodium bicarbonate in distilled water made up to 1 litre**: (v)** Working dilution for coating antigen; 1:3 in tripling dilutions downwards. **(vi)**Working dilution for sera; 1:40 in doubling dilutions across the plate**.(vii)** Working dilution for conjugate; 1:1000.**(viii)** Substrate; used as supplied**;** Stop solution; 1% SDS, diluted in dH_2O. Two 96-well Nunc Maxisorp® ELISA plates were used. Columns 2-10 were coated with 100µl of RVF antigen and coating buffer in the format: Antigen was serially diluted downwards in coating buffer at a ratio 1:3, so that row A had 1:3 (⅓ antigen and ⅔ coating buffer) and row H 1:6561 (Figure 1).

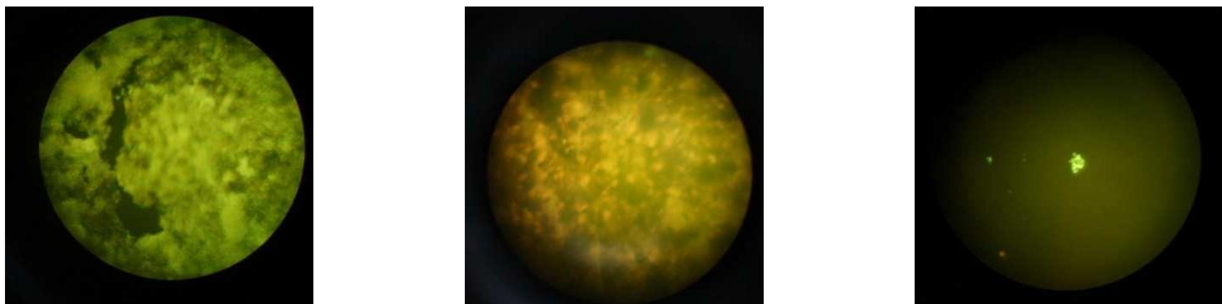
The serial dilution involved placing of 100μ l buffer in all wells and adding 50μ l of Antigen in wells of row A from col. 2-10, then mixing them 5 times using a pipette, and transferring 50μ l of the mixture from row A to row B, until row H. The final 50μ l picked was discarded. Columns 11 and 12 had no antigen added to them, and so acted as controls for the procedure, as did wells in column 1 which were left blank. The plates were covered with lids and incubated overnight at 4ºC, after which they were flipped to rid off the uncoated antigen and washed thrice with PBST (Phosphate Buffer Saline and 0.1%Tween20). 200µl of blocking buffer was added to all wells and plates incubated at 37ºC for 1hour. Plates were flipped and washed in 3 parts of PBST. Positive sheep sera and Negative sheep sera suspended in serum Diluent buffer were coated in 1:2 serial dilutions thus:(i) 1:40 dilutions of positive and negative sera were made in separate 15ml falcon tubes by placing 4000μ l of Diluent buffer in each tube, removing off it 100µl and replacing the 100μ l with positive and negative sera respectively to yield a $^{1}/_{40}$ concentration of sera. (ii) Wells in column 2 were added to them 200µl each of the 1:40 positive sera. Wells in column 3-6 were added to them 100µl Diluent buffer. A 1:2 serial dilution was done by picking 100µl positive sera from column 2 and placing it in column 3, then after mixing 5 times, picking another 100µl from col.3 and placing in col. 4 until col.6. 100µl picked from col.6 is placed in col.11 and mixed. The 100µl from col.11 is discarded. (iii) Wells in column 7 were added to them 200µl each of the 1:40 negative sera. Wells in col. 8-10 were added to them 100μ l of Diluent buffer. A 1:2 serial dilution was done by picking 100μ l of negative sera from col.7 and placing it in col.8, then after mixing 5 times, placing 100µl of the mixture in col.9 until col.10. The 100µl picked from col.10 is place in col.12 after which the final 100μ l picked is discarded.(iv) Col.1 wells were coated with Diluent buffer only. No sera were added. Plates were gently shaken and incubated at 37ºC for 2 hours after which they were flipped and washed in 4 parts PBST. 100μ l of sheep anti-RVF HRPO conjugate was added to wells in col.2-12. Wells of col.1 instead had 100µl PBS added to them. The plates were covered in aluminium foil and incubated at 37ºC for 1 hour after which they were washed in 4 parts of PBST. 100μ l of substrate, ABTS was added to all wells. Plates were shaken briefly, covered in aluminium foil and incubated at room temperature in a dark place for 30mins. The reaction (hydrolysis of ABTS by HRPO which causes green colour development in positive wells) was stopped by adding 100μ l 1% SDS solution in all wells. Reading of optical density was done at 405nm.

Results

The Vero cell cultures were successfully cultured *in vitro* and infected with RVF virus which showed 10% CPE (Figures 1a)

Figures 1a: Left, clean growing Vero cell cultures, and right, RVF-infected Vero cells showing cytopathic effect (CPE)

in just over 3 days. Using the IFA test that utilised monoclonal antibody for RVF antigen, it was confirmed that the CPE observed was a result of RVF virus infection (Figures 1b).



Figures 1b: IFA test observations-left: positive control; centre: test slide with the RVF infected Vero cells; and right, negative control from supernate derived from the spun RVF infected Vero culture

The third objective was to determine standard concentrations of RVF antigen and sheep anti-RVF antibody from the optical density (OD) readings of the most accurate ELISA plate, which are depicted in the figures 2 and 3.

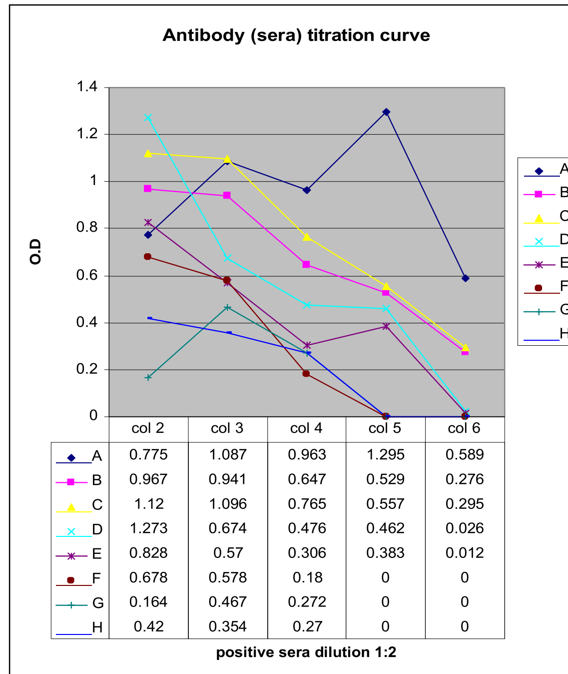


Figure 2: Antibody Titration curve

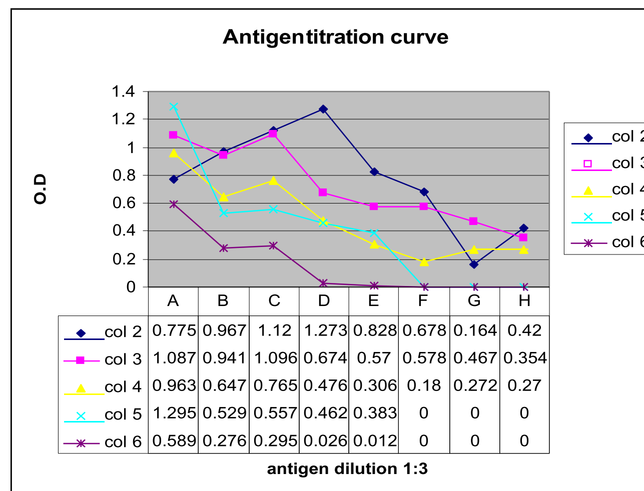


Figure 3: Antigen Titration Curve

The curve in Figure 2 represents the best curve of distribution as it transcends from OD reading of over 1 to less than 0.5. The well, C3, gave the optimal OD reading of 1.096, and this reflected the optimum concentrations of antibody (positive sera of Sheep H2550) to be 1:27. The curve in Figure 3 represents the best curve of distribution and gave the optimal concentration of antigen as 1:80 from well C3.

Sera dil. 1:2

Antigen Dil. 1:3	1	2 1:40	3 1:80	4 1:160	5 1:320	6 1:640	7 1:40	8 1:80	9 1:160	10 1:320	11 1:1280	12 1:640
A 1:3												
B 1:9												
C 1:27												
D 1:81												
E 1:243												
F 1:729												
G 1:2187												
H 1:6561												

Figure 4: Plate layout of ELISA Titration Chequerboard

KEY:

Col.1; Blank: no antigen, no conjugate

Col. 2-6; positive sera [Sheep H2550]

Col. 7-10; negative sera [2524 Pre-inoculum]

Col.2 & 7; no Diluent buffer

Col.11; no antigen, lowest dilution of positive sera

Col.12; no antigen, lowest dilution of negative sera

Figure 4 shows the plate layout of ELISA titration chequerboard, while

Table 1: Dispensation of Trypsin and RPMI-1640 media

	Vol. of Trypsin swirled (5 secs)	Vol. of Trypsin incubated (2min)	Vol. of RPMI-1640 media
T25 flask	4ml	2ml	8ml
T75 flask	6ml	3ml	20ml
T175 flask	8ml	4ml	35ml

Table1 shows the apparatus required for the dispensation of Trypsin and RPM -1640 media. Curves were constructed using Microsoft Excel® 2003 software to identify the curve that transcends the widest range of OD readings (Figures 2 and 3).

Discussion

The success in culturing and preserving RVF virus in Vero cell cultures substantiated earlier information (Craig *et al.,* 1967) that RVFV can be grown in cell cultures and stored in culture fluid at 4ºC and will retain infectivity for 30 days without loss of titre.

The cultures in this study were grown over a period of one and a half months and the virus was preserved at -20ºC over the culturing period. On inoculation, the RVF virus proved competent in infectivity hence showing that the live attenuated RVF virus is stable at -20ºC. During the culture of Vero cells, the importance of supplying CO_2 was appreciated as lack of it resulted in depreciated growth of Vero cells. CO_2 was taken up by cells to maintain the bicarbonate buffer system that was essential for pH regulation. Occurrence of bacterial and fungal contaminations challenged the culture process too. Contaminated cultures were discarded aseptically. During the passaging process, the importance of passaging confluent cells in order, from 25cc to 75cc to 175cc was appreciated. Overstretching of passage lines resulted in much slower growth, and cells affected had to be boosted by increasing FBS in the culture to 20%. Observation of 10% CPE within 4 days fell just outside the known range. According to El Mekki and Van der Groen (1981), viral antigens may be detected in cell cultures in as little as a few hours up to 3 days post inoculation and cytopathic effect (CPE) can be observed 2 to 3 days after antigen detection. The small disparity can be attributed to use of a live- attenuated virus with low virulence, as well as repeated passaging which increases sensitivity of cell cultures (Drosten *et al.,* 2003). The feasibility of detecting the RVF virus from *in vitro* cultures was confirmed. This concurred with earlier information that a tissue culture system combined with the fluorescent antibody technique can provide a rapid means of diagnosis for RVF (El Mekki and Van der Groen, 1981).

In confirming presence of the RVF virus using the IFA method, it was learnt that the virus at 10% CPE, is largely located inside the cells (intracellularly), rather than extracellularly. When IFA test was conducted on the cell pellet and supernated (from spun 10% CPE culture), introcytoplasmic fluorescence was observed from the pellet and virtually no fluorescence from the supernate. This observation also confirmed that centrifuging the 10% CPE Vero cells at 2500rpm for 15 minutes does not destabilise the RVF virus and does not lyse the cells. The ELISA readings showed a general trend in decrease of OD across the plate for the antibody titration and vertical decrease in OD for the antigen titration. This signified that the optimisation was accurate and sensitive to differing concentrations of the antigen and antibody. The OD readings revealed that concentrations of Ag and Ab often differ (the antigen was more concentrated than the antibody) giving credence to optimisation processes before making diagnostic kits. The standard concentrations of antigen and antibody can be applied in sero-diagnosis using Inhibition ELISA (Paweska *et al.,* 2005b).

Conclusion:
The culture of Vero cells, *in vitro* multiplication of RVF virus and optimisation of antigen and sheep anti-RVF antibody was successfully conducted in less than 60 days with results obtained from the standardisation process being 1:80 for antigen and 1:27 for antibody. These concentrations would form the basis of designing a local diagnostic kit.

Recommendations:
The study recommended validation of the optimised antibody and antigen concentrations by subjecting them to an ELISA-based diagnostic study of a wide range of sera samples from different species and localities. Such validation would give credence to the competence of the optimised concentrations in designing an ELISA-based diagnostic kit. Further optimisations and validations of the developed diagnostic kit will enable safe and affordable *en masse* production for commercial purposes, especially within the areas vulnerable to Rift Valley fever, to be realised.

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