CHAPTER 3

CLONING AND EXPRESSION OF THE RIFT VALLEY FEVER VIRUS GLYCOPROTEIN GENES IN A BACTERIAL SYSTEM AND DEVELOPMENT OF A RT-PCR

1 INTRODUCTION

To identify the infectious agent in a suspected Rift Valley fever (RVF) case involve serological tests as well as culturing of the virus from infected samples collected during the febrile stage of the disease (OIE Manual, 1996). A disadvantage associated with the culturing of virus is that it is time consuming and virus-infected material has to be handled. Because RVF is zoonotic, handling of virus-infected material presents a health risk. Diagnosis based on serology include neutralisation tests ($PRNT_{80}$), Enzyme-linked Immunosorbent assay (ELISA), Heamagglutination inhibition (HI), Agar gel immunodiffusion (AGID), Immunofluorescence (IF), Radio-immuno assays (RIA) and complement fixation (CF). Although cross-reactions can occur while performing these tests (Swanepoel, *et al.*, 1986), such assays have the advantage of being performed using an inactivated antigen (OIE Manual, 1996; Swanepoel, *et al.*, 1986). Currently used ELISA assays can distinguish between very early infection and late infection as well as previous contraction or contact with the infectious agent by means of detecting circulating IgM or IgG antibodies using respectively a capture ELISA or a double sandwich ELISA (Niklasson, *et al.*, 1984). Disadvantages of these particular ELISA assays include the use of whole virus as antigen, low sensitivity, high cost and complexity because of the inclusion of several steps for detection of IgG or IgM.

In developing an alternative ELISA or alternative assay to assist in RVF diagnosis, use of a recombinant viral antigen, higher sensitivity, less complicated procedures and a more affordable assay would be preferred. A PCR diagnostic assay can assist in diagnosis and confirmation of the disease. It would have a high sensitivity and would allow differentiation between vaccinated and naturally infected animals in conjunction with serological techniques. PCR assays would have a quantitative function in evaluation of production of classical vaccines, in specific quality control thereof. Such

an assay could in time replace the ELISA based assays as main diagnostic test for diagnosis and confirmation of RVF and quality control of vaccine manufacturing procedures.

Alternative antigens for use as vaccines or to function in ELISA tests include subunits of the viral entity, whether recombinant or synthetic. Options in producing recombinant proteins include bacterial vector systems; baculovirus vector systems as well as Yeast vector systems (i.e. *Pichia pastoris*) for expression of the protein subunits. Because the bacterial expression system is simpler, expression can be regulated by cloning under the control of inducible promoters and expression levels of the antigen can be relatively higher than obtained in other systems, it may be used to produce recombinant antigen for use in an ELISA format assay. A disadvantage of this system is that downstream processing of the protein, including glycosylation, may not be correct or will not occur at all. The resulting gene product may be dysfunctional because of disruption or destroying of epitopes. Solubility of the antigen may also be low due to production of the antigen in the inclusion bodies in the bacterial cells. A system like the baculovirus expression system or the *Pichia pastoris* expression system can provide functions for post-translational modifications. Although the antigen will then be expressed in lower levels than that of the bacterial system, post-translational modification (i.e. glycosylation) would give a product similar to the natural protein. The expressed product might have a higher degree of solubility than the bacterial expressed protein.

In attempting to generate an alternative antigen to the currently used whole virus, the glycoprotein genes of RVFV were cloned into a bacterial expression system. The pPROEX-HTb bacterial expression vector was used to clone the glycoprotein encoding genes of the Rift Valley fever virus (RVFV) in. From this cloned segment, both glycoproteins can be expressed using the accompanying translational start sites. After induction of expression, protein products were examined by electrophoresis and subsequent western blotting using a RVFV specific antiserum.

A one-step RT-PCR assay was developed, optimised, evaluated and validated. Such an assay would have several useful applications as discussed including early and rapid diagnosis of the infectious agent in serum and whole blood. A one-step method was developed instead of a two-step method or a method where a nested reaction is

included. This means a more user-friendly procedure that eliminates unnecessary events where contamination is more likely to occur. Primers were designed to anneal in the intergenic region between the genes encoding the glycoproteins (G1 and G2) on the M segment of the RVFV genome. These docking sites allow the detection of the viral M segment. Additional docking sites would be on the S segment of the virus RNA encoding the nucleocapsid protein. However, since these primers were also used to detect the plasmid DNA vaccine during development thereof, the M segment proved to be the region of choice for primer docking sites. The specificity and sensitivity of the RT-PCR was determined and sera from animals that participated in a DNA vaccination trial and field samples (sheep sera) positive for RVFV obtained from Senegal was used to validate this test.

2 MATERIALS AND METHODS

2.1 Subcloning and expression of the Rift Valley fever virus glycoprotein genes

2.1.1 Subcloning of the RVFV glycoprotein genes into the pPROEX-HTb bacterial expression vector.

The *Nco* I/*Eco* RI fragment containing the glycoprotein genes was excised from the pSCRV-6 plasmid (see Chapter 2, Fig. 1) using enzymes supplied by New England Biolabs. The digestions were done using reaction conditions as indicated by the supplier. The subsequently gel-purified (Talent) fragment was directionally cloned into the pPROEX-HTb (Fig. 15) bacterial expression vector (Life Technologies) into the *Nco* I/*Eco* RI restriction sites, ensuring the inserted fragment to be in-frame with the translational start codon contained in the expression vector. An amount of 5U of the enzymes was used in the reactions in a final volume of 10 µl. The subsequent ligation reaction was performed in a final volume of 15 µl and 1.5 U T4 DNA ligase (Roche) was used with the appropriate amount of supplied buffer.

The pPROEX-HTb bacterial expression vector contains the sequence that codes for six histidine affinity tags to facilitate subsequent purification of the expressed protein. A Tobacco Etch virus (TEV) protease recognition site allows for removal of the histidine tags by protease cleavage from the expressed protein during purification (Fig. 15). The

histidine affinity tag would be located on the N-terminal of the expressed product. Expression of the protein from the genes cloned into the pPROEX-HT bacterial expression system is facilitated by isopropyl-β-D-thiogalactopyranoside (IPTG) induction (0.6 mM final concentration). Insertion of the gene fragment was verified by restriction digestion of the obtained recombinants. The confirmed correct recombinant plasmid was digested with *Mlu* I (AEC-Amersham) and analysed by electrophoresis on a 1% agarose gel stained with ethidium bromide.

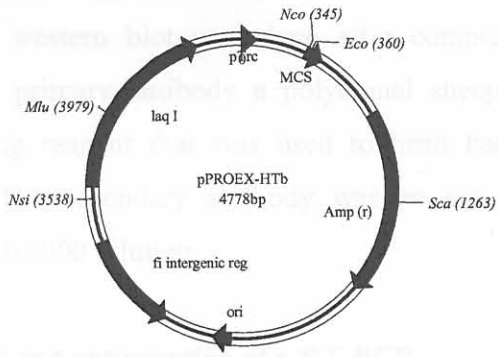

Figure. 15 - Diagrammatic representation of pPROEX-HTb bacterial expression vector indicating the multiple cloning site of the vector used to clone the glycoprotein gene fragment of RVFV excised from pSCRV-6.

2.1.2 Expression of the glycoprotein genes from the bacterial expression system

After verification of the suspected recombinants using restriction enzymes, a single colony of the stock culture streaked onto Luria-Bertani (LB) agar plate, was picked with a sterile toothpick. This was inoculated into 10 ml LB-broth and incubated at 37 °C. After the culture has reached a cell density of 0.5-1.0 at A_{590}, a 1 ml sample was removed and after pelleting the cells, it was resuspended in phosphate buffered saline (PBS). This sample served as the uninduced control. Isopropyl-β-D-galactopyranoside (IPTG) was subsequently added to the culture at a final concentration of 0.6 mM as indicated by the

accompanying plasmid product manual supplied by Life Technologies. Aliquots of 1ml of the induced cultures were removed at 3h post-induction, the samples were centrifuged and to the pellets were added PBS and an equal volume of sodium dodecyl sulphate (SDS) sample buffer (125 mM Tris-HCl (pH. 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 0.01% (w/v) bromophenol blue). Samples were taken in duplicate of which one received Dithiotrietol (DTT) (10 mM final concentration) added to the SDS sample buffer. The treated samples were stored at –70 °C until use. Samples were boiled and analysed by SDS-polyacrylamide gel electrophoresis on a 12% gel where 20 µl of the processed sample was loaded onto the gel. The supernatants as well as the pellets were treated in the same way. A western blot was done after completion of the electrophoresis procedure, using as primary antibody a polyclonal sheep-anti-RVF antibody diluted 1/100. The blocking reagent that was used to limit backgound was bovine serum albumine (BSA). The secondary antibody was an anti-sheep antibody coupled to peroxidase used at a 1/1000 dilution.

2.2 Development and optimisation of a RT-PCR

2.2.1 Designing of primers

Primers were designed to anneal at positions 1755-1775 and 2097-2117 for the forward (ARV1-F) and reverse (ARV2-R) primers respectively, on the M segment of the RVFV genome (Collet, *et al.*, 1985). Both primers were synthesized by Gibco BRL at an amount of 200 nmole each. The position where primer ARV1-F anneals, is at the 3' end of the G2 glycoprotein gene whereas ARV2-R anneals in the intergenic region between G2 and G1. The part that is being amplified is mainly the intergenic region. Sequences for ARV1-F and ARV2-R are 5' -tgt cac act gct ctc agt gcc–3' and for ARV2-R, 5' –gga gct tgc ctg aat ctg ttg-3' respectively. The amplicon size expected, after amplification of the template using these primers is 363 bp. Self-complementarity and alignments between the primers were checked as well as specificity for the template using the DNAMANSoftware (Version 2.6; Lynnon Biosoft).

2.2.2 Use of RVFV-specific primers in PCR reaction

A PCR was done using the plasmids pSCRV-6 and pCI-G2G1-EGFP as templates at an estimated 3ng dsDNA each to confirm the specificity of the primers for the template sequences. The pCI-G2G1-EGFP plasmid was also used at a 1:10 dilution of the plasmid. The composition of the reaction mixture was as follows:

Forward primer (ARV1-F)	(50 pmole/µl)	0.5 µl
Reverse primer (ARV2-R)	(50 pmole/µl)	0.5 µl
Takara ExTaq	5U/µl	1.0 µl
Supplied buffer (Takara)	10x	2.5 µl
dNTP's (Roche)	10 mM	1.0 µl
Template		3 ng
Water to a final volume of		25 µl

Magnesiumchloride ($MgCl_2$) was not added additionally to this reaction volume, thus the final concentration was 2.5 mM as included in the supplied buffer. The time-temperature profile of the reaction was 30 cycles of 94 °C for 30 sec; 61 °C for 30 sec; 72 °C for 30 sec.

2.2.3 Extraction methods for template RNA

Rift Valley fever virus (Smithburn strain used for live attenuated viral vaccine production) was obtained from Onderstepoort Biological Products and used to serve as template source during the optimisation of the RT-PCR. The virus has been passaged in mice 103 times, twice in Baby Hamster Kidney (BHK) cells and once in African Green Monkey kidney (VERO) cells to attenuate the virus. This attenuated virus stock that was obtained had a titre of 10^6 TCID/ml and was aliquotted and stored at –20 °C.

Two RNA extraction methods were evaluated. These were the QIAmp viral RNA extraction kit from Qiagen, and the method described by Chomczynski, *et al.*, (1987). Both methods were used according to the protocols described by either the manufacturer or the author respectively. Briefly, for the QIAmp viral RNA extraction kit, 140 µl of the naïve blood sample was used. To the sample was added the buffer containing carrier

RNA and absolute ethanol. This was loaded onto a column containing a silica-gel membrane supplied with the kit and centrifuged to retain the RNA in the column. The column was washed with the supplied buffers and the RNA eluted using the supplied elution solution. In the case of the method described by Chomczynski, *et al.,* (1987), 200 µl of the sample (serum or blood) was added to an equal volume of solution D (4M Guanidiumthiocyanate, 25 mM sodium citrate, 0.25 % Sarcosyl, 100 mM 2-mercapto ethanol) and 20 µl 2M NaAc. This was mixed and 500 µl phenol: chloroform (1:1) was added. After incubation on ice, the sample was centrifuged and the supernatant was added to 500 µl isopropanol before incubation at –20 °C to facilitate precipitation of the RNA. After centrifugation, the RNA pellet was washed with 70 % ethanol and then redissolved in 50 µl Diethylpirocarbonate (DEPC) treated water.

Blood was spiked with an estimated amount of 10^2 $TCID_{50}$ of the virus per extraction volume. The extractions were performed and the RNA obtained was stored for a short time at –20 °C until use. Naïve serum was also spiked with the virus to determine the possibility of extracting virus from blood and serum. A one-step RT-PCR method was performed with the following time-temperature profile. 37 °C for 30 min; 94 °C for 30 sec; **65 °C** for 30 sec; 72 °C for 30 sec in a 25 µl reaction volume. 30 cycles were completed. The annealing temperature was raised from 61 °C to 65 °C to increase specificity of the reaction. Reaction volumes had the following reagent composition :

Forward primer	(AVR1-F)	(50 pmole/µl)	0.5 µl
Reverse primer	(ARV2-R)	(50 pmole/µl)	0.5 µl
MMLV-RT (Promega)	200U/µl		0.1 µl
Ex Taq (Takara)	5U/µl		0.25 µl
Ex Taq buffer	10x		2.5 µl
dNTP's (Roche)	10mM		1.0 µl
HPRI (Promega)	110U/µl		0.01 µl
Template RNA			5 µl
Water to a final volume of			25 µl

Final concentration of $MgCl_2$ in this reaction was 2.0 mM included in the buffer.

2.2.4 Magnesiumchloride titration

A $MgCl_2$ titration was done to determine optimum $MgCl_2$ concentration to be used in the RT-PCR reaction. Final concentrations used in the titration assay were 0 mM; 1.5 mM; 2.5 mM; 5 mM; 7.5 mM and 10 mM. The titrations were performed in serum-extracted samples using the method of Chomczynski, *et al.*, (1987) after this method has been chosen as the preferred extraction method due to lower cost and ease of use. In this titration the annealing temperature was raised to 65 °C as was done for the reactions in which the extraction methods were compared. This would allow for higher specificity.

2.2.5 Sensitivity determination of RT-PCR

Sensitivity of the assay was determined by diluting the virus (titre of 10^6 $TCID_{50}$/ml) in naïve blood and serum samples. The total volume of sample for the extraction method needed to be 200 µl (Chomczynski, *et al.*, 1987). Dilutions of the virus were done in 100µl PBS and added to 100µl blood or serum. The amount of virus (Smithburn attenuated viral vaccine stock of 10^6 $TCID_{50}$/ml) added was 50µl, 25µl, 12.5µl, 6.25µl, 0µl respectively to an amount of PBS for a final volume of 100µl. This was then added to the 100 µl naïve serum or blood for a final volume of sample of 200 µl. Dilutions of virus and addition of virus to serum was also repeated after serially diluting the virus in PBS. The final dilution was 10^{-6} and the virus titre predicted for this dilution was 1 $TCID_{50}$. The RNA was extracted from these virus dilutions in serum or blood. The RT-PCR reaction was repeated as described for the magnesiumchloride titration.

2.3 Validation of the developed one-step RT-PCR.

The samples used in validation of this one-step RT-PCR assay were serum samples obtained from the sheep that have been injected with the DNA vaccine and challenged with a virulent strain of RVFV. The specific sera tested in this experiment were taken on day three post-challenge, since it is from day 2-5 that maximum virus titres

are expected for RVFV. RVF positive field samples obtained from Senegal were tested using the one-step RT-PCR protocol described here (results not shown). All the serum samples obtained from animals participating in the DNA vaccination trial were stored at –70 °C after preparation until used for RNA extraction using Chomczynski's method (Chomczynski, *et al.*, 1987). The one-step RT-PCR reaction was performed using the same conditions as for the sensitivity assay and magnesiumchloride titration previously described. The sheep were bled at day 3 and day 6 after challenge with the virulent viral strain and the serum samples were obtained from blood collected in this way.

3 RESULTS

3.1 Subcloning glycoprotein genes in the pPROEX-HTb bacterial expression vector

The glycoprotein genes were cloned directionally into the multiple cloning site of the pPROEX-HTb bacterial expression vector using the *Nco* I/ *Eco* RI restriction enzymes. Following cloning the gene fragment into the vector digested with these enzymes, the gene product should be expressed as a fusion protein with the six histidine tags at the N-terminal of the protein.

In doing small-scale plasmid DNA isolation of the recombinant plasmids, it was observed that the plasmid DNA yields obtained was very low in comparison to small-scale plasmid DNA isolations done from cultures containing the pPROEX-HTb vector alone. To be able to visualise the plasmid DNA extraction product on an ethidium bromide stained agarose gel, the amount needed to be loaded onto a gel was five times more than the amount needed for other constructs (Fig. 16) (see Chapter 2 as well). Insertion of the gene was verified by restriction digestion of the recombinant plasmid (7.7 kbp) with an enzyme having a unique restriction site in the inserted glycoprotein gene fragment and not in the 4.7 kbp vector (agarose gel not shown).

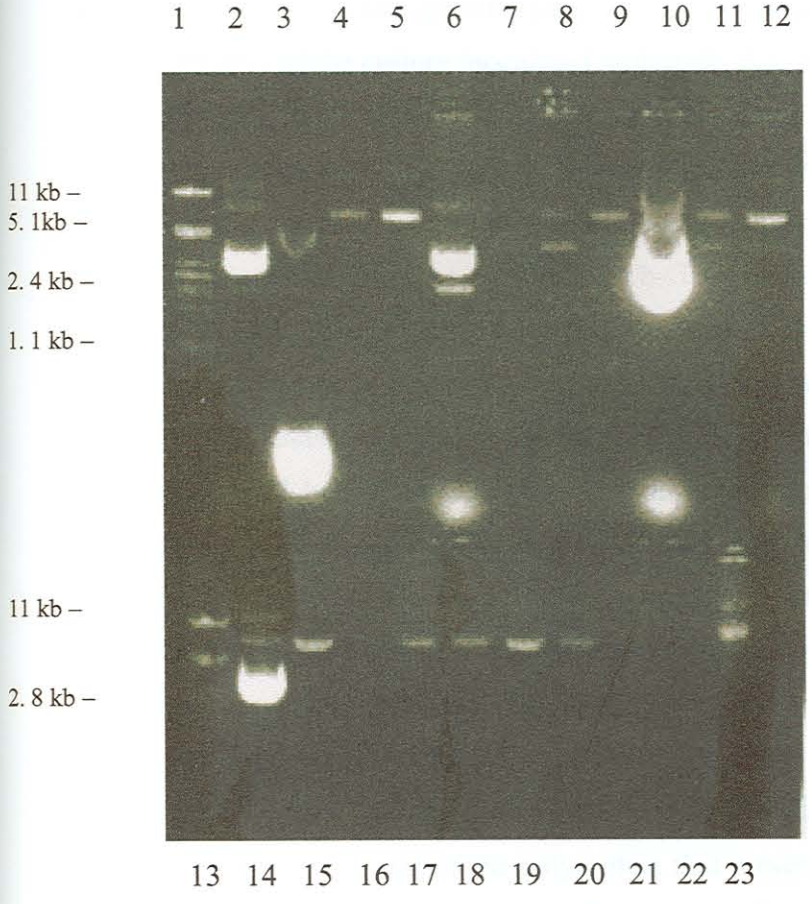

Figure. 16 Comparison of plasmid DNA yields obtained in culture containing recombinant pPROEX-HTb-G2G1 plasmid and the vector plasmid respectively. The first lanes (1 and 13) in the top and bottom row represent the lambda DNA digested with Pst I to serve as molecular weight marker. Next to the marker (lane 2 and 14) was loaded the vector control and in the remaining lanes the transformants picked from the solid medium and cultured overnight in 3 ml LB-broth (3 to 12 and 15 to 23).

3.2 Expression of the glycoprotein genes

After inoculation of the medium with pPROEX-HTb-G2G1 transformants, the cell density had to be between 0.5 and 1 AU at 590nm in order to ensure that the cells are in the logarithmic growth phase before inducing with IPTG. The absorbancy (cell density) of the culture inoculated with pPROEX-HTb-G2G1 increased much slower and reached the desired cell densities much later than the culture inoculated with pPROEX-HTb vector control. The vector control reached an absorbance of 0.5 after 4h30 min. A sample was taken from the vector control (non-induced vector control) after which the IPTG was added. The pPROEX-HTb-G2G1 culture reached an absorbancy of 0.5 after 6h30 min when the expression of the inserted glycoproteins could be induced. A non-induced control sample was taken from this culture before addition of IPTG. Two hours after induction the absorbance value of the test culture have not increased and even when incubated longer, no significant rise in absorbance values could be detected. Samples were taken to analyse for expression of the glycoprotein genes on an SDS-PAGE gel (Fig. 17). Electrophoresis profiles of the expression products on SDS-PAGE gels showed no detectable additional protein products at the expected size locations of the G2 and G1 glycoproteins to the vector background proteins after induction with IPTG. However, at the position of between 105 and 160 kDa, a faint band could be visualised in the lanes that represent the samples collected after induction and incubation of the induced sample overnight. The size of the polyprotein that presents the primary translation product was observed to be 130 kDa. In performing a western blot, it would be possible to detect if the protein represented by this band, is RVFV specific.

In an attempt to generate an alternative antigen for use in an ELISA based diagnostic assay, another inducible bacterial expression system has been used to examine expression of the glycoproteins from the RVFV M segment. The pRSET bacterial expression system (Invitrogen) is a phage-facilitated system that upon infection with the helper phage and induction with IPTG, drives expression of the genes cloned under the control of the inducible promoter present in the plasmid vector. The full-length fragment encoding the G2 and G1 glycoprotein genes as well as a truncated version of the G2 glycoprotein gene were cloned into the vector (Charlotte Ellis, Marie Hamman; Applied Biotechnology, Onderstepoort Veterinary Institute). From the clone containing the full-

length form of the M segment, no expression products could be obtained after induction (results not shown). However, when the truncated form of the G2 glycoprotein was expressed, a clear band at the expected molecular weight of 30 kDa could be detected. At this stage, this expressed antigen is being evaluated as alternative antigen in an ELISA based diagnostic assay against RVF.

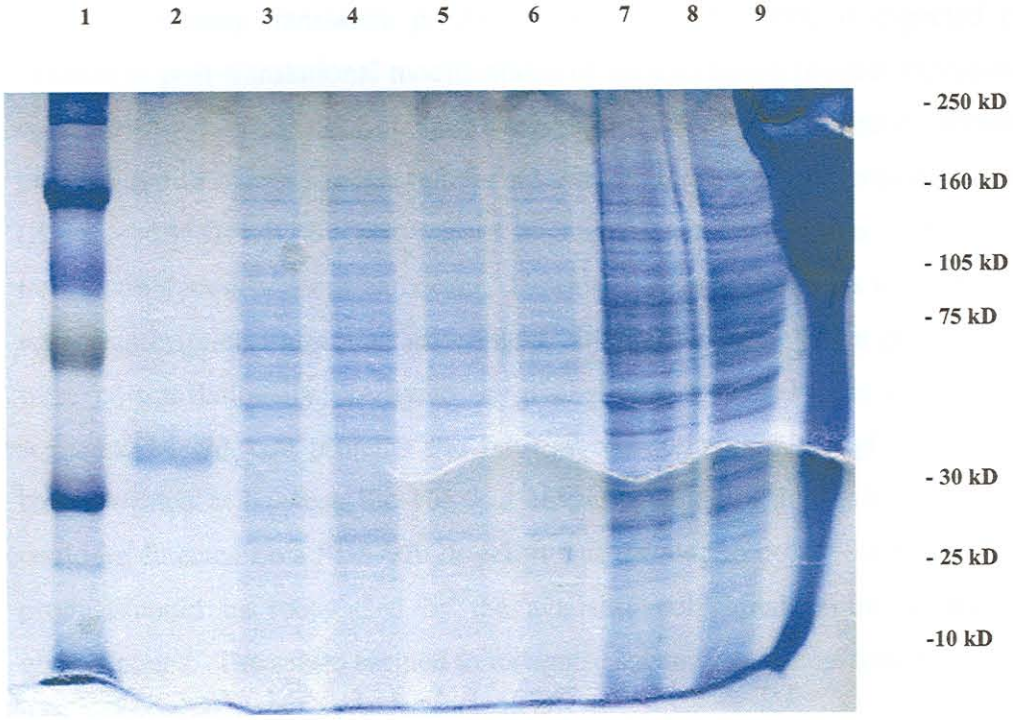

Figure. 17. SDS-PAGE gel with proteins expressed from pPROEX-HTb in *E. coli* liquid culture after induction with IPTG, separated on a 12 % SDS-polyacrylamide gel stained with Coomassie. The pellets were treated with PSB. The left lane (1) represents the molecular marker (kD) (Amersham). In lane 2 were loaded a truncated form of the G2 glycoprotein of RVFV expressed in the pRSET bacterial expression system. To the samples in lanes 3, 5 and 7 were added 15 mM DTT and the samples in lanes 4, 6 and 8 no additional DTT was added in the PSB. In lane 9 was loaded 20 µl of 10^6 pfu RVFV (Smithburn attenuated strain) in 200 µl PBS. Lanes 3 and 4 are the pre-induced culture, lanes 5 and 6 the 1hour post-induction samples and lanes 7 and 8 the overnight post-induction samples.

Sizes of the glycoproteins are respectively 65 and 56 kDa for G1 and G2 (Collet, *et al.*, 1987; Kakach, *et al.*, 1989). Because glycosylation is not expected to take place in the bacterial expression system, the glycan moieties would be absent causing the molecular weight of both proteins to be smaller, 53kDa and 57kDa respectively (Keegan and Collet, 1986), and the electrophoretic mobility of the proteins in a SDS-PAGE gel tends to increase (Kakach, L.T. *et al.*, 1989).

A primary translation product (Kakach, *et al.,* 1989) is expected due to the absence of post-translational modifications of the expression product expressed from the open reading frame (ORF) of the M segment of RVFV in a bacterial system. Such a product of 133 kDa was observed during a study of *in vitro* transcription and translation (Kakach, 1989). It did not appear in a eukaryotic expression system where post-translational modifications can occur. However, when microsomes were present in the *in vitro* transcription-translation experiment, this primary translation product of 133 kDa were co-translationally processed to give all the expected gene products of the M segment (14, 78 kDa proteins and both glycoproteins). Although only the additional bands located at between the 105 and 160 kDa bands in the molecular weight marker could be detected on a SDS-PAGE gel, it was decided to do a western blot to see if any product could be recognised by the available polyclonal serum against the RVFV glycoproteins. The serum seemed to recognise several bands non-specifically (Fig. 18).

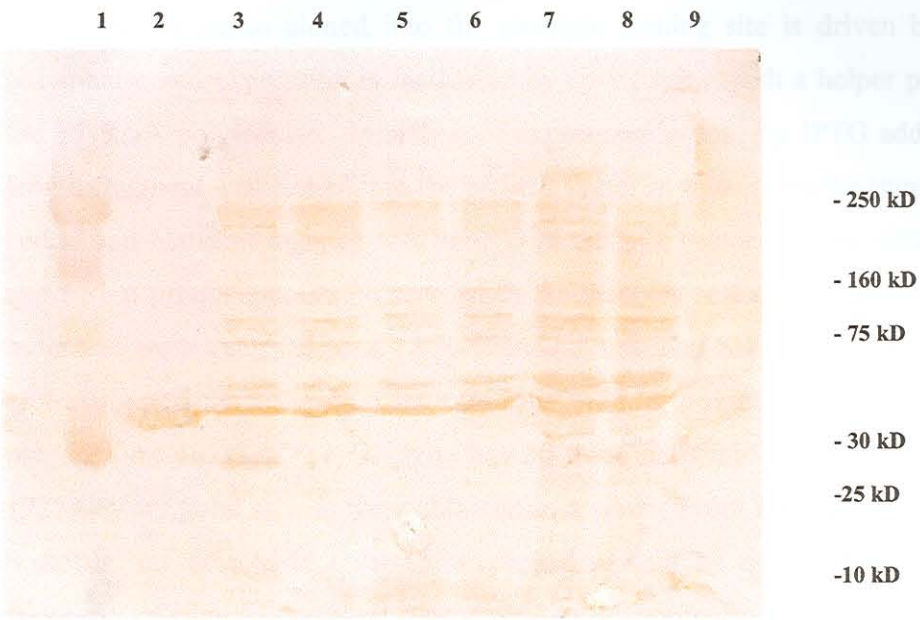

Figure. 18 Western blot of the proteins in pellets of *E. coli* cultures transformed with pPROEX-HTb pre-and post-induction with IPTG and treatment with DTT. The left lane (1) represents the molecular marker (kD) (Amersham). In lane 2 were loaded a truncated form of the G2 glycoprotein of RVFV expressed in the pRSET bacterial expression system (Invitrogen). To the samples in lanes 3, 5, and 7 were added 15 mM DTT and the samples in lanes 4, 6 and 8 no additional DTT was added in the PSB. In lane 9 was loaded 20 µl of 10^6 $TCID_{50}$ RVFV (Smithburn attenuated strain) in 200 µl PBS. Lanes 4 and 5 are the pre-induced culture, lanes 5 and 6 the 1hour post-induction samples and lanes 7 and 8 the overnight post-induction samples. The blot was blocked with 10 % albumine, developed with anti-RVFV serum (1/100) and the Protein G conjugated to peroxidase at a dilution of 1/1000 used as conjugate.

In the pRSET bacterial expression system, the sequence coding for six histidine molecules is 5' of the multiple cloning site in which the gene fragment had been cloned. Expression of genes cloned into the multiple cloning site is driven by the T7 RNA polymerase and expression is facilitated by co-infection with a helper phage expressing the T7 RNA polymerase. Induction of expression is done by IPTG addition. The full-length fragment was cloned into the pRSET vector in frame with the translation initiation codon and histidine tags, as was done with the pCI vector and the pPROEX-HTb. No expression products could be seen when the samples collected after time intervals after induction were analysed on a 12 % Coomassie stained SDS-PAGE gel or on a western blot using polyclonal serum (Charlotte Ellis and Marie Hamman). This encouraged us to use the first 43 % of the G2 gene having three antigenic determinants (Collet, *et al.*, 1987). Very good signals were obtained in a western blot for the truncated form of G2, however, no detectable levels of expression could be obtained with the full-length fragment of the M segment encoding the glycoprotein genes, whether in pRSET (Charlotte Ellis and Marie Hamman, results not shown) or pPROEX-HTb.

3.3 Development of a RT-PCR

The primer pair was found to be specific in annealing to the glycoprotein gene fragment. When using computer software to determine self-complementarity of the primer pair to itself, no self-complementary regions were found. Recognition of the primers to plasmid template at an annealing temperature of 61 °C were successful in giving an amplicon of the correct size of 363 bp (Fig. 19) and in the PCR reaction no non-specific amplification of background bands were observed.

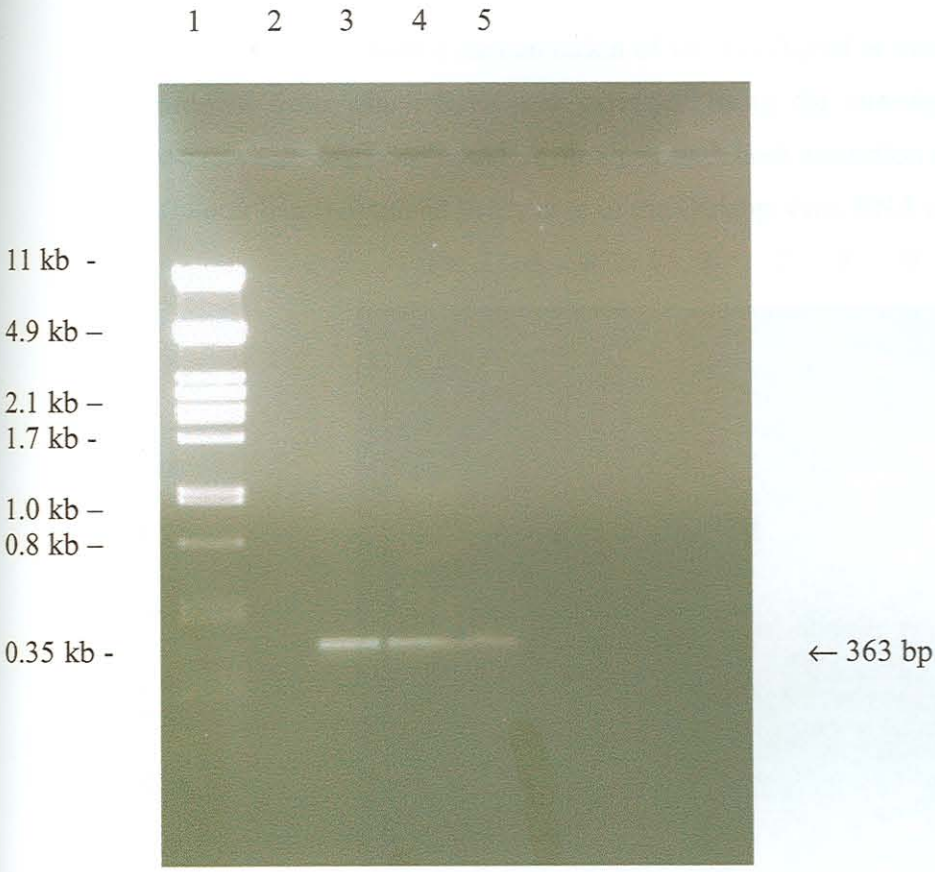

Figure. 19 The PCR amplicons obtained after using plasmid material as template to determine the specificity of the primers for the glycoprotein gene sequence. Lane 1 - molecular weight marker (λ DNA digested with Pst I), lane 2 - water control, lane 3 - pSCRV-6 as template, lane 4 - pCI-G2G1-EGFP as template, lane - 5 1:10 pCI-G2G1-EGFP. Amplicon size is 363 bp. A 1% agarose gel was used and the DNA was vizualised using ethidiumbromide.

Template RNA could be obtained by using extraction methods described by Chomczynski *et al.* (1987) and the Qiagen manufactured QIAmp Viral RNA extraction kit (Fig. 19). It was decided to use the one-step isolation method of Chomczynski for subsequent extraction procedures for economic reasons, availability of reagents, ease of use and quality of the obtained product. The samples used to compare the RNA extraction methods were naïve sheep blood spiked with 100 µl of the Smithburn

attenuated virus with a concentration of 10^6 $TCID_{50}$/ml or virus material only of the same amount from which RNA was extracted using the one-step extraction method. The correct size amplicon could be obtained with both extraction methods, with a less intense (fainter band) obtained in the case of the QIAmp Viral RNA extraction kit.

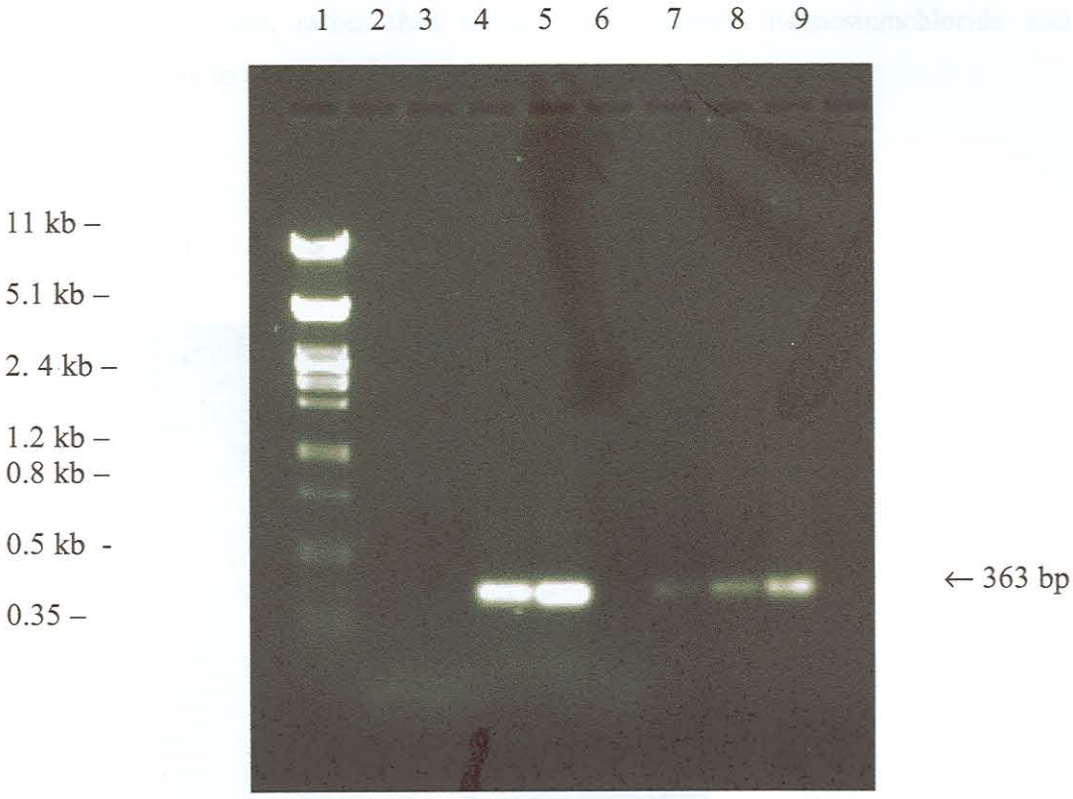

Figure.20 Comparison of extraction methods used to extract RNA from blood artificially spiked with the virus stock and virus stock (Smithburn attenuated virus strain) only. Lane 1-(λ DNA digested with Pst I), lane 2- water control; (lanes 3-5 with method described by Chomczynski, P. *et al.*, (1987) lane 3 - naïve blood; lane 4 - blood spiked with virus stock; lane 5 - virus stock; (lane 6 - 8 QIAmp viral RNA extraction kit) Lane 6 - naïve blood; lane 7 - spiked blood with virus stock; lane 8 – virus stock only (10^6 $TCID_{50}$/ml); lane 9 – plasmid DNA as positive control. Analysis done on a 1% agarose gel, stained using ethidiumbromide.

A titration was done to determine the optimal magnesiumchloride concentration in the RT-PCR. A concentration of 2 mM was found to be to be optimal in the RT-PCR reaction (Fig. 21). Since the concentration of magnesiumchloride present in the supplied enzyme buffer is 2.5 mM it was decided to keep the concentration at 2.5 mM in the reaction volume, rather than using buffer without magnesiumchloride and adding additionally to the reaction volume.

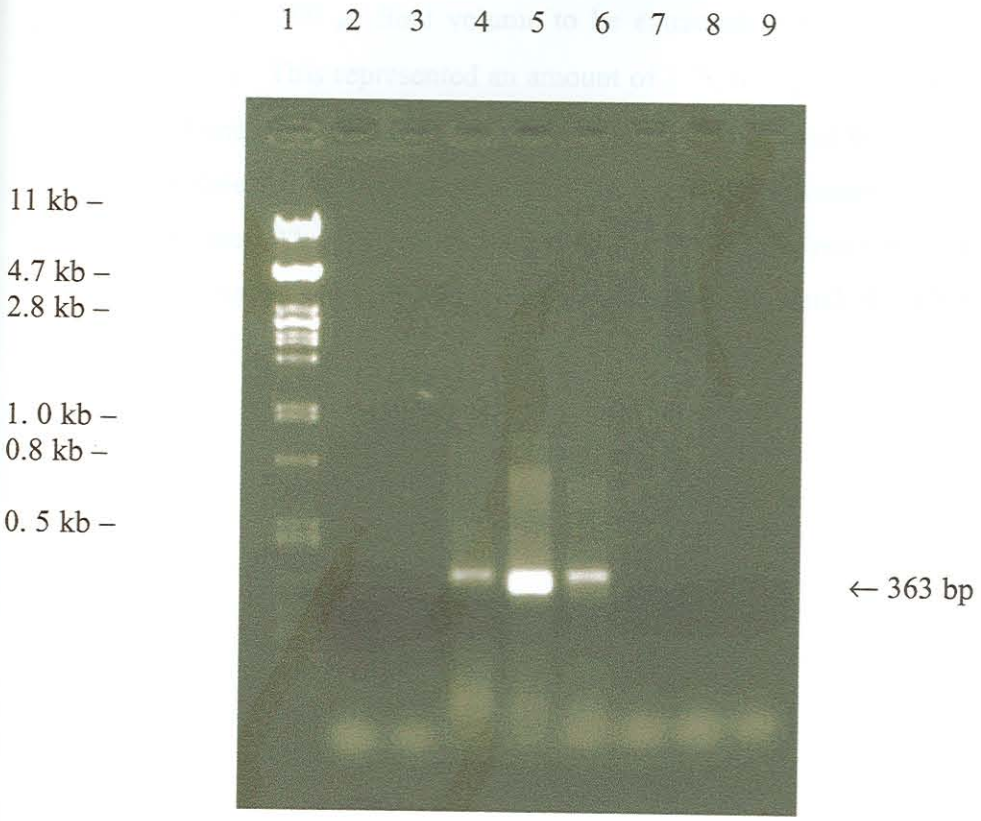

Figure 21 Titrations were done to determine the optimal concentration of magnesiumchloride to be used in the one-step RT-PCR reaction. Lane 1- Lamda DNA digested with Pst I as molecular weight marker. Lanes 2- Water control, 3- 0mM, 4- 1.5mM, 5- 2mM, 6- 2.5mM, 7- 5mM, 8- 7mM, 9- 10mM. Analysis was done on a 1% agarose gel, DNA visualised with ethidiumbromide.

A serial dilution of the Smithburn attenuated virus strain (10^6 $TCID_{50}$/ml) was done from 10^6 $TCID_{50}$ to 1 $TCID_{50}$ in serum and blood. At this stage no amplification product could be obtained from spiked blood. This compares to the results that were obtained in the comparison of extraction methods, where the products obtained were of a low yield when the QIAmp Viral RNA extraction kit was used to extract RNA from blood spiked with the virus. The absence of an amplicon is unclear but might be explained by the presence of a PCR inhibitor like haemoglobin. The faintest band of amplification product could be detected at a dilution of $5x10^{-4}$ (1 µl of the 10^{-3} virus dilution, 200 µl final volume to be extracted; 100 µl virus dilution in PBS + 100 µl serum). This represented an amount of 1 $TCID_{50}$ present in the volume extracted (20 µl volume of extracted material) of which 5 µl was used from the total of 20 µl, that could be detected in the one-step RT-PCR assay. The amount of viral RNA included in the one-step RT-PCR reaction amounts to 0.25 $TCID_{50}$/reaction. This gives an indication of non-viable virus present in the virus sample of which the RNA could still be detected (Fig.22).

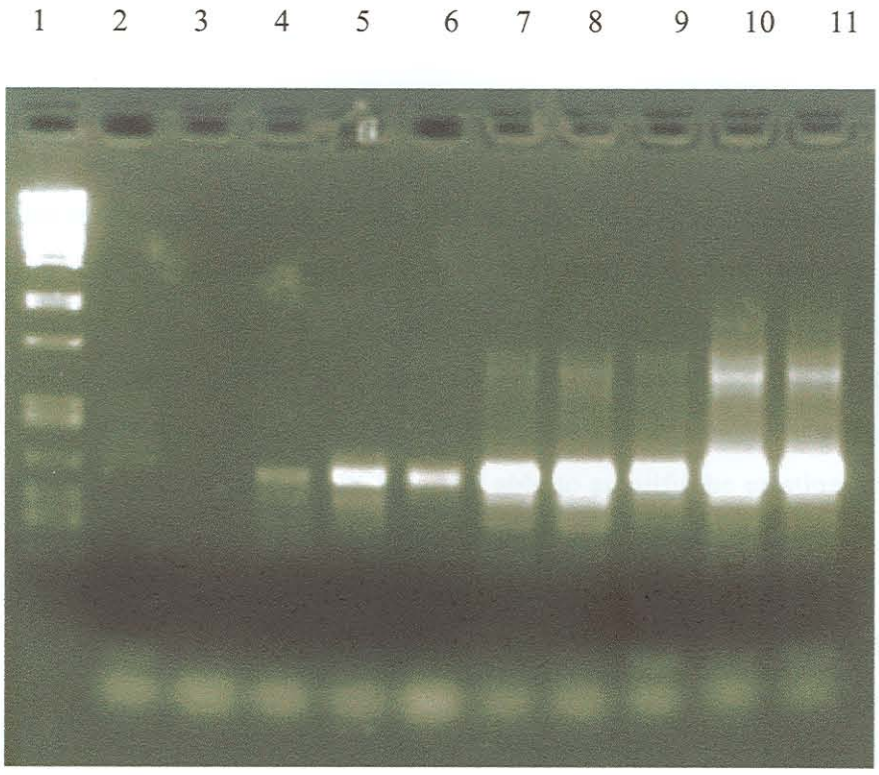

Figure.22 Titration of Smithburn attenuated virus material in serum to determine the relative sensitivity of the RT-PCR. Lane-1, Molecular weight marker; Lane-2, Water; Lane-3, Negative contol; Lane-4, 1/1000 dilution; Lane-5, 1/500 dilution; Lane-6, 1/100 dilution; Lane-7, 1/50 dilution; Lane-8, 1/10 dilution; Lane-9, 1 µl virus material diluted into 100 µl PBS and added to 100 µl serum; Lane-10, 6.25 µl virus material into 100 µl PBS added to 100 µl serum; Lane-11, only virus material (no further dilution into serum). DNA visualised on the 1% agarose gel using ethidiumbromide.

In order to validate the developed one-step RT-PCR procedure, sera were obtained from sheep participating in the DNA vaccination trial (for individual animal data see table 3, chapter 2). Amplicons could be obtained in all animal samples (Fig. 23). The result obtained in this case is an indication that virus template was detected in the vaccinated animals after challenge, indicated the presence of replicating virus. Since the positive controls in this experiment were vaccinated with an attenuated virus, replication of the viruses injected in the challenge dose in these animals is limited and thus much lower. However, the RT-PCR is still able to amplify the existing virus RNA. A further application of the RT-PCR might be the evaluation of a vaccine. To evaluate the DNA vaccine (Chapter 2) as well as other vaccines using the developed RT-PCR protocol, the aim would be to test sera obtained daily after challenge with a virulent strain and using this to monitor the virus clearance from the blood in this way. It would be expected that the vaccine eliciting higher levels of protection would lead to more rapid clearance of the virus from the blood.

These results indicate sufficient quantities of virus replicated in all animals for the RT-PCR to detect, whether vaccinated with the attenuated virus or the DNA vaccine. The intensities varied in some of the bands and this might be due to different levels of virus replication, even though differences in template amounts due to bias in the extraction procedure cannot be ruled out.

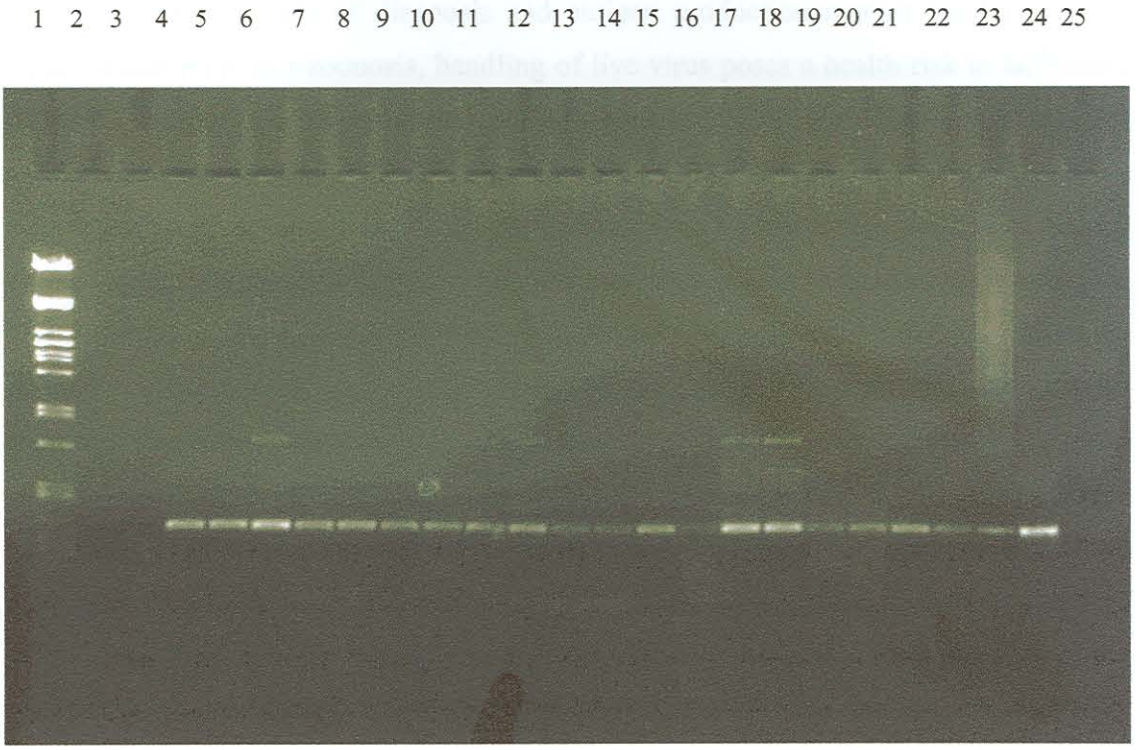

Figure.23 RT-PCR amplicon products obtained from serum samples of animals that participated in the DNA vaccine trial (injection with PCI-G2G1-EGFP) and subsequent challenge with a virulent strain of RVFV. The sera tested and shown in this figure were taken on day 3 post-challenge. The positive control used was viral spiked serum (virus titre of 10^3 $TCID_{50}$), indicated in lane 24. Lane 1 - molecular weight markers (Lambda DNA digested with Pst I), lane 2 the water control, lane 3 - negative serum, lane 4 - 3345, lane 5 - 3670, lane 6 - 3004, lane 7 - 3671, lane 8 - 3373, lane 9 - 3372, lane 10 - 3283, lane 11 - 3762, lane 12 - 3678, lane 13 - 3650, lane 14 - 3383, lane 15 - 3673, lane 16 - 3347, lane 17 - 3313, lane 18 - 3643, lane 19 - 3287, lane 20 - 3010, lane 21 - 3368, lane 22 - 3648, lane 23 - 3764 (Addendum V and VI list the animals that participated in the DNA vaccination trial, additional samples - sheep vaccinated with the "clone 13 " attenuated virus as positive controls).

4 DISCUSSION

Current methods of diagnosis and antigen production require handling of the virus. Since RVF is a zoonosis, handling of live virus poses a health risk to laboratory workers. A substitute for the whole virus as antigen would be recombinant viral proteins or viral protein subunits. The glycoproteins of RVFV is situated at the outside of the virus particle, it is antigenic and immunogenic. Since neutralising antibodies can be elicited against it, the glycoproteins should be useful in an ELISA-based diagnostic assay. The generation of such a recombinant viral protein can be done in different expression systems. These include eg bacterial expression systems, Baculovirus expression system and yeast expression systems.

The main advantage of bacterial expression systems is ease of use. On the other hand, the expressed proteins will not be glycosylated correctly due to the absence of post-translational modifications in prokaryotic cells. It was observed for the cloned genes of RVFV, that solubility of the expressed proteins is a problem due to expression of the proteins in the inclusion bodies found in the prokaryotic cell (Collet, *et al.,* 1987). Solubilising of the proteins using detergents may destroy epitopes on the protein or interfere with the subsequent downstream applications of the antigen.

In an attempt to generate recombinant viral antigen (glycoproteins) the glycoprotein gene fragment was subcloned in the pPROEX-HTb bacterial expression plasmid as well as in the pRSET phage-facilitated bacterial expression system (results not shown). No expression products could be detected after subcloning the RVFV glycoprotein gene fragment into the pPROEX-HTb. The cells were harvested and lysed 3 hours post-induction. Induction was done by adding 0.6 M IPTG. The supernatants (cell lysates) as well as the cell pellets were analysed on a 12% SDS-PAGE gel (Fig. 17). No additional protein bands representing the glycoproteins G2 and G1 were seen when compared to the negative vector control. In a western blot (Fig. 18), antibodies in RVFV-specific polyclonal sheep serum did not bind to proteins of sizes similar to G1 and G2 in either the cell pellet or the supernatant. However, it was observed that the *E. coli* culture transformed with pPROEX-HTb-G2G1 reached a cell density of 0.5 AU at 590 nm two hours after the *E.coli* culture transformed with pROEX-HTb after inoculation at the same

time. In addition, the plasmid DNA yield obtained of pROEX-HTb and pPROEX-HTb-G2G1 differed markedly (Fig. 16). These differences could be caused by an inhibiting effect of the subcloned gene on the replication of the plasmid, leading to lower yields in comparison to the vector alone. The expressed products might have a toxic effect on the cells, explaining the lower growth rate. However, it was seen in cloning the truncated version of the G2 glycoprotein, that the plasmid yield and growth rate of cells transformed with the resultant construct, compares to that of bacterial cells transformed with plasmid only.

Subcloning of the glycoprotein gene fragment into the Baculovirus pFASTBAC helper plasmid and subsequent expression of the gene product could result in a product more soluble (Schmaljohn, *et al.*, 1989) but a lower yield in comparison to the bacterial expression system. Although it is expected that post-translational modifications take place, it is not a given fact for each gene product. The bacterial system was chosen as first option, because it might have been possible that the expressed antigens have the capability to present the epitopes recognized by the RVFV specific antibodies in a way comparable to the natural situation (Keegan, *et al.,* 1986).

Subcloning the glycoprotein gene fragment into the pRSET expression plasmid, also did not result in detectable expression of G2 or G1 (see Chapter 3, results). However, the truncation of the M segment fragment lead to the expression of a truncated form of the G2 glycoprotein containing 43% of the N-terminal part of the G2 protein. Currently this product is being evaluated as recombinant RVFV antigen in an ELISA format assay. In preparation of this antigen, no effect on bacterial growth patterns could be detected as in the case of the full-length cloned fragment. The exact cause and level of influence of the full-length gene fragment or gene products of the full-length fragment in the bacterial system on the growth of the cells and copy number of the plasmid remains to be determined.

The one-step RT-PCR that was developed, evaluated and partially validated is sensitive enough to detect 0.25 $TCID_{50}$ in RVFV positive sera samples from which RNA has to be extracted (200 µl, equal thus to 1 TCID50/ml of infected sample). Maximum viremic titres of the virus observed in adult sheep were $10^{7,6}$ $MIPLD_{50}$/ml (Coetzer, J.A.W., *et al.,* 1994), indicating that diagnosis of the disease using this RT-PCR would be

possible. None of the related *Phleboviruses* causes disease in sheep that might be confused with RVF based on serology (Swanepoel, *et al.,* 1986). Thus in conjunction with serology, the diagnosis of RVF would have a high specificty. Since there was no outbreak in the area, field samples are not available to add to the validation data at this stage. RVFV positive samples obtained from Senegal were available to use in the one-step RT-PCR assay. Of these samples tested, some were found to be positive in the ELISA based diagnostic assay, were found to be negative when using the one-step RT-PCR. This could be explained by the fact that virus RNA was degraded in the samples at the time of RNA extraction since the samples were already repeatedly freeze-thawed. The one-step RT-PCR assay was also performed on the serum samples obtained from the sheep injected with pCI-G2G1-EGFP and challenged with a virulent RVFV. Amplicons of the expected size were obtained in all of the samples at varying intensities. This is an indication of RVFV present in sufficient levels to detect with the RT-PCR, even in vaccinated animals. However, a lower viral load than obtained in the negative control would give an indication of the ability of the vaccine to protect against the virus disease due to the immunological response that is being elicited.

The fully validated and evaluated test can be used as a diagnostic assay. Quantitative tests for quality control of classical vaccine production can be done using this RT-PCR assay. For discrimination between vaccinated and naturally infected animals, it would be necessary to design the primers according to the vaccine being tested for at that specific time. Diagnosis and differentiation would be done at this stage in conjunction with serology.