DEVELOPMENT OF A RECOMBINANT ANTIGEN FOR THE DETECTION OF ANTIBODIES AGAINST RIFT VALLEY FEVER VIRUS IN HUMANS AND ANIMALS

Petrus Jansen van Vuren

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Promoter: **Prof. A.A. Van Dijk**
North-West University, School for Biochemistry, Potchefstroom

Co-promoter: **Dr. J.T. Paweska**
National Institute for Communicable Diseases, Special Pathogens Unit, Sandringham

Co-promoter: **Dr. A.C. Potgieter**
Onderstepoort Veterinary Institute, Biochemistry Division, Onderstepoort

North-West University, Potchefstroom Campus
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ABSTRACT

Rift Valley fever (RVF) is a mosquito-borne zoonotic disease endemic to Africa that presents a significant health threat to both humans and animals. Outbreaks have serious economical implications. Two facts cause concern. Firstly, recent outbreaks in the Arabian Peninsula and effects of global warming have the implication that the virus might spread further into non-endemic RVF regions since it utilizes a wide range of mosquito vetors. Secondly, RVFV is a potential bio-terrorism agent. Therefore, quick, safe, robust and cost-effective methods of detection and appropriate diagnostic reagents are needed for early diagnosis, disease surveillence and bio-defense field monitoring. The development of several effective ELISAs for the detection of antibodies to RVFV have been reported (Paweska et al., 2003a, 2003b and 2005a; 2005b). A serious drawback of these ELISAs is that they use an inactivated virus as antigen. The availability of a recombinant antigen would lower the safety risks of antigen preparation and the costs of diagnosis. The purpose of this study was to clone and express a recombinant RVFV nucleoprotein (N) from a southern African RVFV strain (Zim 688/78), and to evaluate its suitability as a diagnostic antigen in an ELISA for the detection of antibodies to RVFV in humans and animals.

The gene encoding the N protein was amplified by RT-PCR using primers specifically designed to contain restriction enzyme sites for cloning. The nucleic acid sequence of the gene was determined and compared to that of the 7 other published N gene sequences of different strains. The N gene of the Zim688/78 strain had 5 unique nucleic acid differences, all in wobble positions which did not cause amino acid changes. The nucleic acid sequence of the Zim688/78 strain was the closest related to the Clone13 strain (98.6%), and its amino acid sequence was identical to Clone 13. A fragment of the gene encoding the glycoprotein G2 was also amplified, its nucleic acid sequence was determined and compared to the published G2 sequences of other strains. The G2 fragment was closest related to the BEgy93 (98.7%), HEgy93 (98.7%) and ZH548 (98.7%) strains.

The Zim688/78 N gene was cloned into two bacterial expression system vectors, pGEX4T-1 and pET32(a)+ and three baculovirus expression vectors, pFastBac-1, pFastBacHT-B and pBACgus-1. Low amounts of completely insoluble recombinant N protein (rN) were expressed by baculovirus recombinants generated using pFastBacHT-B. The expression level of rN using pFastBac-1 was higher and rN was more soluble. These constructs, including the pBACgus-1 construct, was sent to Biovac (Pinelands, South Africa) for further optimization of baculovirus expression. Bacterial expression using the pGEX4T-1 vector resulted in a high yield of rN, but it was mostly insoluble. However, IPTG induced bacterial expression with the pET32(a)+ construct resulted in a high yield of soluble rN. The recombinant nucleoprotein was purified with Ni-affinity column purification, using the pET32(a)+ fusion tag with 6x His residues.

The purified rN was of high quality and therefore it could be used for direct coating of immunoplates in indirect ELISA (I-ELISA). Various coating procedures were tested. Overnight coating in carbonate/bicarbonate buffer (pH 9.6) on a MaxiSorp (Nunc) plate gave the best results. The recombinant

nucleoprotein antigen based I-ELISA was used to measure IgG and IgM antibodies in titrated sera from vaccinated personnel and naturally infected humans respectively. The test was able to distinguish between sera with high and low concentrations of antibodies. The rN-based I-ELISA was also used to measure IgG and IgM antibody responses in vaccinated and experimentally infected sheep. The test was able to show seroconversion in both vaccinated and infected sheep, track the rise and decline of IgM antibodies and detect the expected lower levels of the immune response in sheep vaccinated with live-attenuated virus compared to that of sheep infected with wild type virus. The rN also proved to be suitable as an antigen in IgG sandwich and IgM capture ELISA formats.

The production of the RVFV rN under experimental procedures used was quick, easy, relatively inexpensive and safe. The findings of this project demonstrated that the recombinant is a high quality diagnostic antigen for use in I-ELISAs for the detection of antibodies in humans and animals.

OPSOMMING

Slenkdalkoors is 'n virale soönose wat deur muskiete oorgedra word. Dit hou 'n groot ekonomiese en gesondheidsrisiko in vir mens en dier. Die siekte is endemies in Afrika, maar onlangse uitbrake in die Arabiese Peninsula het groot kommer veroorsaak omdat dit gewys het hoe maklik die virus kan versprei na nie-endemiese gebiede. Die virus kan ook moontlik vir bio-terrorisme gebruik word. As gevolg van die bogenoemde is dit nodig om vinnige, veilige en koste-effektiewe metodes te ontwikkel vir die diagnose van slenkdalkoors asook die toepaslike diagnostiese reagense. Verskeie ELISA metodes is reeds ontwikkel vir die opsporing van teenliggame teen die virus (Paweska et al., 2003a, 2003b and 2005a, 2005b;). Die nadeel van hierdie metodes is egter dat hulle gebaseer is op die gebruik van geïnaktiveerde virus as antigeen. The beskikbaarheid van 'n rekombinante antigeen sal die kostes en gesondheids risiko verlaag van die produksie van antigeen en die diagnose van infeksie met die virus.

Die doel van hierdie studie was die klonering en uitdrukking van 'n rekombinante nukleokapsied (N) protein van 'n suidelike Afrika stam van die virus, Zim688/78. Verdere was die doel om die antigen se toepaslikheid te evalueer as 'n diagnostiese antigeen vir die opsporing van teenliggame in diere en mense. Die geen wat kodeer vir die N proteïen is geamplifiseer met RT-PCR deur gebruik te maak van spesiaal ontwerpte voorvoerders wat herkenningsvolgordes bevat vir restriksie ensieme om klonering te vergemaklik. Die nukleïensuur volgorde van die N geen is bepaal en vergelyk met die N geen volgordes van die 7 reeds gepubliseerde virus stamme. Die N geen van die Zim688/78 stam het 5 unieke nukleïensuur variasies gehad wat almal in "wobble" posisies was en dus nie aminosuur veranderinge veroorsaak het nie. Die nukleïensuur volgorde van die Zim688/78 stam was die naaste verwant aan die "Clone13" stam (98.6%), en hul aminosuur volgordes is ook identies. 'n Fragment van die geen wat kodeer vir die glikoprotein G2 is ook vermeerder, die nukleïensuur volgorde bepaal en vergelyk met die G2 volgordes van ander gepubliseerde stamme. Die G2 fragment was die naaste verwant aan die BEgy93 (98.7%), HEgy93 (98.7%) en ZH548 (98.7%) stamme.

Die N geen is gekloneer in twee bakteriele uitdrukkingsvektore, pGEX4T-1 en pET32(a)+, en drie baculovirus uitdrukkingsvektore, pFastBac-1, pFastBacHT-B en pBACgus-1. Evaluasie van baculovirus uitdrukking van die N proteïen is gedoen met pFastBac-1 en pFastBacHT-B. Die rN produksie met pFastBacHT-B was laag en dit was onoplosbaar. Produksie met pFastBac-1 was hoër en meer oplosbaar. Hierdie konstrukte, tesame met die pBACgus-1 konstruk, is gestuur na Biovac (Pinelands, Suid Afrika) vir verdere optimalisering van die uitdrukking van die rN proteïen.

Bakteriële uitdrukking van die N proteïen met die pGEX4T-1 vektor het goeie produksie gelewer maar die produk was gedeeltelik onoplosbaar. Bakteriële IPTG geïnduseerde uitdrukking met die pET32(a)+ konstruk het baie oplosbare rN produk gelewer. Hierdie rekombinante N protein is gesuiwer met behulp van die 6x His stert wat deur die vektor aan die proteïen geheg is.

Die gesuiwerde rN protein was baie suiwer en is daarom gebruik as antigeen om plate te bedek vir indirekte ELISA (I-ELISA). Verskeie maniere van bedekking van die plate is getoets. Oornag inkubasie by 4°C met karbonaat/bikarbonaat (pH9.6) buffer op MaxiSorp (Nunc) plate het die beste resultate gelewer het. Die rN antigeen gebasseerde ELISA was gebruik om IgG en IgM teenliggame te monitor in verdunningsreekse van serum van ingeënte en geïnfekteerde mense afsonderlik. Die toets het die vermoë gehad om te onderskei tussen serum met hoë en laer konsentrasies van teenliggame. Die rN antigeen gebaseerde ELISA was ook gebruik om IgG en IgM teenliggame te monitor in beide eksperimenteel geïnfekteerde en ingeënte skape. Die toets kon die produksie van teenliggame, die verskyning en verdwyning van IgM teenliggame en die verwagte laer vlakke van immuunreaksie aandui in ingeënte skape in vergelyking met geïnfekteerde skape. Daar is ook bewys dat die rekombinante nukleokapsied antigeen nuttig is vir gebruik in 'n IgG toebroodjie ELISA en IgM vang ELISA formate.

Die produksie van die rekombinante nukleokapsied is maklik, vinnig, relatief goedkoop en veilig. Die bevindinge van die projek is 'n bewys van die beginsel dat die rekombinante nukleoproteïen 'n uitstekende antigeen is vir gebruik in I-ELISAs vir die opsporing van teenliggame teen slenkdalkoors in mense en diere.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Rift Valley fever (RVF) is a mosquito-borne zoonotic disease that presents a significant threat to humans and domestic ruminants in South Africa and other African countries. RVFV is endemic in Africa in the following countries: South Africa, Kenya, Namibia, Mozambique, Zimbabwe and Zambia, and in other African countries such as Senegal, Mauritania, Sudan, Egypt, Chad and Somalia (Anonymous, 2003). An epidemic caused by RVFV has huge economical effects because of severe disease caused in domestic animals. The 1950-1951 outbreak in South Africa resulted in the death of almost 100 000, and abortions of almost 500 000 sheep (Gerdes, 2004; Swanepoel & Coetzer, 2004). Outbreaks of RVF disease occur at irregular intervals and go hand in hand with heavy rains and sustained flooding which facilitate breeding of the mosquito vectors (Swanepoel & Coetzer, 2004). Outbreaks of RVF in non-endemic regions outside of Africa such as Saudi Arabia, Madagascar and Yemen show that the RVFV is capable of easily flourishing outside of its endemic region. This is cause for great concern because the virus has a wide range of mosquito vectors (Gora et al., 2000; Mebus, 1998).

1.1 Virus classification and characteristics

RVFV is an arbovirus, member of the *Phlebovirus* genus in the *Bunyaviridae* family (Gora et al., 2000). The *Bunyaviridae* is a family that consists of spherical shaped enveloped viruses (Yadani et al., 1999). Bunyaviridae contains two serogroups, the sandfly fever viruses and the tick-transmitted uukuviruses. The sandfly fever group is composed of at least 8 antigenic complexes: Bajaru, Candiru, Chilibre, Frijoles, Punta Toro, RVF, Salehabad and an unassigned complex containing 16 viruses (Sall et al., 1997). Other important *Bunyaviridae* include pathogens such as Crimean-Congo, Hantaan and Sin Nombre viruses (Le May et al., 2004). RVFV has a diameter of up to 120 nm with short glycoprotein spikes projecting through the envelope (Olaleye et al., 1992). RVFV is most stable between pH 7 and 9, and it is rapidly inactivated by lipid solvents, UV light and temperature of 56°C and above. RVFV has a number of distinct antigenic molecules including the surface antigens (glycoproteins) and a nucleoprotein antigen (Bishop et al., 1980).

The RVFV genome consists of three single-stranded RNA segments; L, M and S. The L segment, consisting of 6606 bases, has negative polarity and encodes the viral RNA-dependent RNA polymerase. The M segment, consisting of 3885 bases, has negative polarity and encodes the precursor of the viral envelope glycoproteins G1 and G2, a 78-kDa non-structural glycoprotein and a non-glycosylated 14-kDa protein. The S segment consists of 1690 bases. It encodes the viral nucleoprotein, N, and a non-structural protein NSs using an ambisense coding strategy (Ihara, Akashi and Bishop, 1984; Giorgi et al., 1991). The N protein (length: 245 amino acid residues, weight: 27,431 kDa) is encoded by 738 bases of subgenomic viral-complementary mRNA. The NSs protein (length: 265 amino acid residues, weight:

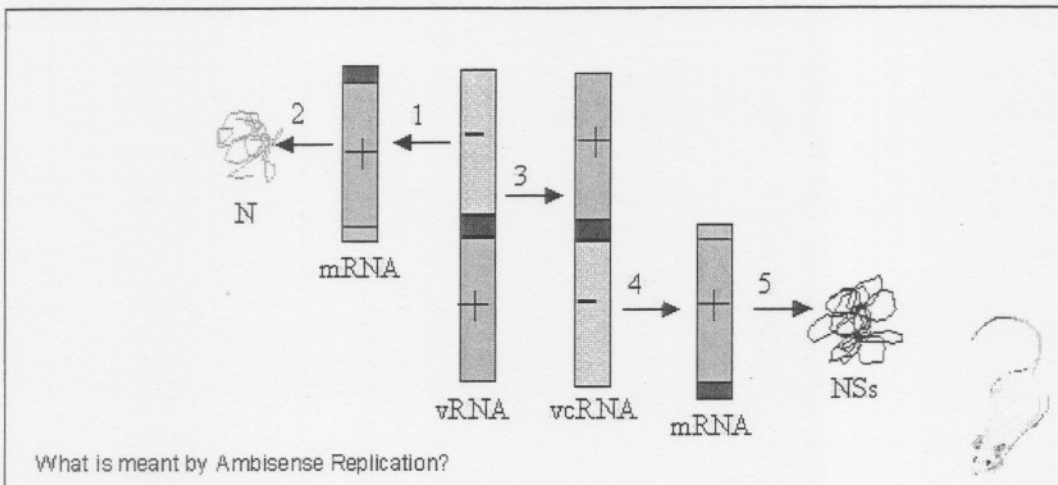
29,903 kDa) is encoded by 798 bases of subgenomic viral-sense mRNA (Billecocq et al., 2004; Rice et al., 1980; Suzich, Kakach, and Collett, 1990).

The ambisense coding strategy of the S segment of RVFV is illustrated in Figure 1. The gene encoding the N protein is negative sense in the 3' half of the viral RNA, which is transcribed into subgenomic viral-complementary mRNA. This mRNA is then translated into the N protein. As the genome is replicated, viral-complementary RNA is formed. The gene encoding the NSs protein is now negative sense in the 3' half of the viral-complementary RNA, which is then transcribed to produce subgenomic viral-sense mRNA. This mRNA is translated into the NSs protein (Giorgi *et. al.*, 1991).

Bunyaviridae genomic RNA molecules from their tripartite genomes contain 5' and 3' terminal non-coding regions (NCR) which are highly conserved in each genus. For *Phleboviruses* these sequences are 8 nucleotides long and they are complementary to each other, which mean that they can form specific panhandle structures (Gauliard *et. al.*, 2006). Even though strains of RVFV differ in their pathogenicity, they are structurally and serologically indistinguishable (Rice *et. al.*, 1980). The gene encoding the N protein of phleboviruses is highly conserved (Giorgi *et. al.*, 1991). Vialat *et. al.* (1997) studied the mutations present in the whole genome of the MP12 RVFV strain. They found mutations in all the genes of the virus except in the gene encoding the N protein, which suggests that the N protein is highly conserved (Vialat *et. al.*, 1997). Further proving that the N protein is highly conserved is the fact that the Clone13 (naturally attenuated) and ZH548 (highly pathogenic) strains differ in only one amino acid at position 159 (Billecocq et al., 2004). Because of this it would be possible to produce a recombinant nucleoprotein with the amino acid sequence of one specific strain that would be able to detect antibodies against any RVFV strain.

Viruses with segmented genomes, including members of the *Bunyaviridae* family, can undergo RNA segment reassortment. This means that two different strains (from the same genus) infecting the same cell can exchange whole segments. Reassortment between RVFV strains has been shown experimentally in tissue cultures and mosquitoes. It was shown (Sall et al., 1999) that natural reassortment occurred between the West African, Central-East African and Egyptian lineages. RNA virus evolution is most commonly caused by replication errors such as deletions or insertions and base substitutions which are only small changes. Major changes in viral genomes may involve exchanges of RNA segments (genetic shift). Studies have shown that evolution of RVFV not only occurs through base substitution (which accounts for only up to 9.6% in S segment) but also through genetic exchange. This could mean that genetic reassortment is a common mechanism in the evolution of RVFV. Mosquito and vertebrate hosts may act as a site for exchange between different RVFV strains, if they are infected with both at the same time (Sall et al., 1999).

Figure 1. The ambisense coding strategy of the S segment of RVFV (Anonymous, 2005).



The NSs is the most variable protein among *Phleboviruses* (Sall et al., 1997). The RVFV NSs protein is different from those of other *Bunyaviruses* in that it is phosphorylated and found in the nucleus of infected cells, which is unique because all *Bunyaviruses* replicate exclusively in the cytoplasm. The NSs protein forms filamentous structures in the host nucleus. In experimental systems NSs have been found to be neither stimulatory nor inhibitory to replication or transcription of RVFV, thus NSs does not play a role in viral growth but has another function. This was proven by Ikegami *et. Al* (2006) who rescued RVFV virus particles from cDNA lacking NSs expression. cDNA that allowed NSs expression inhibited virus rescue. It was suggested (Bouloy et al., 2001) that the role of the NSs protein in RVFV is to inhibit the interferon (IFN) response of the host by blocking the virus-induced IFN-α/β production. This was suggested after finding that RVFV strains that have mutations in the NSs coding sequence (MP12 and Clone13 strains) have an attenuated phenotype, meaning it is not virulent at low concentrations. These viruses induced the production of large amounts of IFN-α/β in their hosts. The ZH548 strain has no mutation in the NSs sequence, is very virulent and does not induce production of IFN-α/β. They also exploited the ability of RVFV to undergo reassortment to develop reassortant viruses. A reassortant carrying the Clone13 L and M segments, but the ZH548 S segment was found to be as virulent as the original ZH548 strain. A reassortant carrying the L and M segments from ZH548 but the S segment from Clone13 was found to be non-virulent and induce IFN-α/β production (Bouloy et al., 2001).

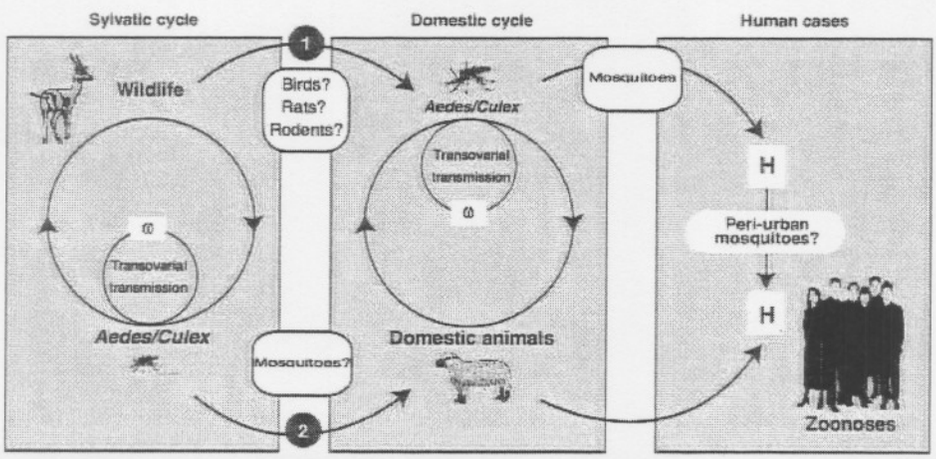
The glycoprotein G2 is important for viral infection and may also play a role in pathogenesis. According to Keegan and Collet (1986) this protein is able to induce the production of virus neutralizing and protective

antibodies against the virus when it is used as an immunogen. Since this protein induces virus neutralizing antibodies, it could be used as a vaccine to protect animals (Keegan and Collet, 1986). The nucleoprotein and viral polymerase proteins of negative sense RNA viruses associate with the virus genome and form ribonucleoproteins (RNPs) which are necessary for transcription and replication (Gauliard *et. al.,* 2006). RNP molecules appear circular because of the complementary sequences of 5' and 3' non-coding regions which cause the formation of panhandle structures (Le May et al., 2005). The nucleoprotein is the most abundant protein in infected cells during any *Bunyavirus* infection (Gauliard *et. al.,* 2006). The N protein has the ability to form oligomers with itself which plays a very important role in transcription and replication of the virus (Le May et al., 2005). The N protein is also the first viral protein to be synthesized, as it is synthesized only 20 minutes post-infection, whereas NSs is only synthesized 60 to 80 minutes post-infection (Ikegami et al., 2005). The net charge of the RVFV nucleoprotein at neutral pH is +9 (Giorgi et al., 1991).

1.2 Aetiology and Epidemiology

RVFV is transmitted mostly by *Aedes* and *Culex* mosquitoes, but other mosquitoes (*Anopheles*, *Eretmapoites*, and *Mansonia*) have also been shown to be successful vectors of the virus (Gora et al., 2000; Mebus, 1998; Sall et al., 1999). This arbovirus can survive in nature for several years in infected mosquito eggs (Le May et al., 2004). RVFV has epizootic and interepizootic cycles. During the epizootic cycle the virus circulates between the arthropod vectors and its hosts (mostly cattle or sheep), where it is amplified. This occurs with unusual heavy rainfall, floods, climate changes or any disturbances in the environment which may increase the number of available vectors. During the interepizootic cycle, mainly dry seasons, the virus survives in transovarially infected mosquito eggs, which produces RVFV infected mosquitoes when they hatch (Sall et al., 1998). The cycle of RVFV transmission can be seen in Figure 2. Analysis of a RVFV strain isolated from a fatal case in the 1997-1998 outbreak in East Africa showed close relation to a strain isolated in Madagascar during the 1990-1991 outbreaks. This proves that this virus can spread over considerable distances.

Figure 2. RVFV transmission cycle (Geering et. al., 2002).



Rift Valley fever virus was first isolated in 1931 in Kenya near Lake Naivasha from the blood of a newborn lamb, after which the first human infections were reported (Daubney and Hudson, 1931). The first report of extensive human disease caused by RVFV was in 1951 in South Africa where an estimated 20 000 persons were infected. In 1977/1978 it was estimated that between 18 000 and 200 000 people were infected in Egypt, of which 598 died from encephalitis and/or haemorrhagic fever (El-Akkad, 1977). The 1977/1978 Egypt outbreak was the first outbreak in a country that is not sub-Saharan, and was the result of the Aswan dam being built six years earlier which created many hectares of flood lands. The Egypt outbreak was characterised by retinitis in a large proportion of the population. An outbreak of RVFV in Mauritania in 1987 was the cause of approximately 200 deaths. This was also the result of the construction of a dam, the Diama dam, along the Senegal River. In 1991 outbreaks in Madagascar and Eastern Africa caused 89 000 infections and more than 500 deaths. In 1997-1998 an outbreak occurred in Kenya and Somalia that affected 98 000 people, of which 250 died (Gerdes, 2004; Sall et al., 1998). This outbreak coincided with the 1997/1998 El Niño year which created an inland lake because of the flooding in Somalia and Kenya. This created the ideal environment for the RVFV mosquito vectors to breed. The most recent outbreak was in Saudi Arabia and Yemen in 2000/2001. It was the first documented occurrence of RVF outside of Africa. During this outbreak 882 cases were documented of which 124 were deaths (Balkhy and Memish, 2003). Large outbreaks in cattle have also been reported in Zimbabwe and Zambia, with an annual emergence in Zambia after seasonal rains indicated by monitoring sentinel cattle. Although there have been no RVFV outbreaks in smaller African countries such as Mali, Gabon, Congo, Chad, Botswana, Angola, Nigeria and Uganda, antibodies to the disease have been found in humans and livestock in these countries (Davies et al., 1992). Figure 3 displays the origins and dates of isolation of RVFV isolates.

The pathogenicity of RVFV may be attributed to the filamentous structures formed by the NSs protein. The virus replicates in the cytoplasm, but the NSs viral protein forms filamentous structures in the host cell nucleus. It is, therefore, believed that this protein disrupts transcription in the host. This is done to evade a response from the host. This is a method utilized by cytopathic RNA viruses that replicate in the cytoplasm and do not need the host enzymes for transcription. TFIIH is a basal transcription factor involved in DNA repair and cell cycle regulation and may be a target for the viral protein. The NSs protein has been shown to interact with the p44 subunit of TFIIH blocking its assembly. This inhibits its helicase activities and the phosphorylation of RNA polymerase II. The cellular concentration of TFIIH, and accordingly RNA synthesis, has been shown to drop with RVFV infection (Le May et al., 2004).

Figure 3. Origins and dates of isolation of RVFV isolates during major RVF outbreaks (Sall et al., 1999).

Code	Strain	Yr of isolation	Origin	Source	Genotype[a] (S/M/L)
SNS[b]	Smithburn	1944	Uganda	Entebbe strain	C/C/C
ArUGA-55	Lunyo	1955	Uganda	Mosquito	C/C/C
ArCAr-69	Ar B 1976	1969	CAR	Mosquito	C/C/C
C13	Clone 13	1974	CAR	Human	*/C/C
HEGY-77	ZH 548	1977	Egypt	Human	E/E/E
MP12[b]	MP12	1977	Egypt	ZH548 strain	E/E/E
ArMAD-79	Ar Mg 811	1979	Madagascar	Mosquito	E/?/E
ArSEN-84	Ar D 38661	1984	Senegal	Mosquito	E/W/W
AnGUI-84	An K 6087	1984	Guinea	Bat	W/C/C
ArBUF-84	Ar D 38457	1984	Burkina Faso	Mosquito	W/W/W
H1MAU-87	H D 47502	1987	Mauritania	Human	C/W/W
H2MAU-87	H D 47311	1987	Mauritania	Human	W/W/W
H3MAU-87	H D 47408	1987	Mauritania	Human	W/W/W
H4MAU-87	H D 48255	1987	Mauritania	Human	W/?/W
AnMAD-91	An Mg 990	1991	Madagascar	Bovine	C/C/C
ArSEN-93	Ar D 104769	1993	Senegal	Mosquito	C/C/W
AnSEN-K-93	An D 106417	1993	Senegal	Zebu	W/W/C
BEGY-93	B EGY 93	1993	Egypt	Buffalo	E/E/E
HEGY-93	H EGY 93	1993	Egypt	Human	E/E/E
HKEN-97	384-97.1	1997	Kenya	Human	C/C/C

[a] E, W, and C, Egyptian, Western African, and Central-East African lineage, respectively; *, sequence too short to be assigned a lineage; ?, sequence not determined.
[b] Attenuated strain.

1.3 Effects on humans

An outbreak in Egypt in 1977 changed the pattern of RVFV from being only a veterinary problem to also being a threat to humans (Sall et al., 2001). Humans are highly susceptible to infection. Transmission of RVFV to humans can occur through a bite from an infected mosquito, contact with contaminated meat or through aerosols created during slaughtering. RVFV has a half-life in excess of 77 minutes at 25ºC and 30% relative humidity in aerosols (Mebus, 1998).

Human infection may take on four forms: i) Uncomplicated, febrile, influenza-like illness; ii) hemorrhagic fever with liver involvement, thrombocytopenia, icterus and bleeding; iii) encephalitis following a febrile episode with confusion and coma or even death; iv) ocular involvement with reported blurred vision and loss of visual acuity due to retinal haemorrhage and macular oedema. The more severe complications occur in up to 5% of the cases. Examination of blood in the presence of RVFV reveals leucopaenia, elevated blood enzymes because of liver damage and thrombocytopenia. The main sites of viral replication are the liver, spleen and the brain. The incubation period of RVFV ranges from 12 hours to six days in young and adult humans, with illness lasting seven to eight days.

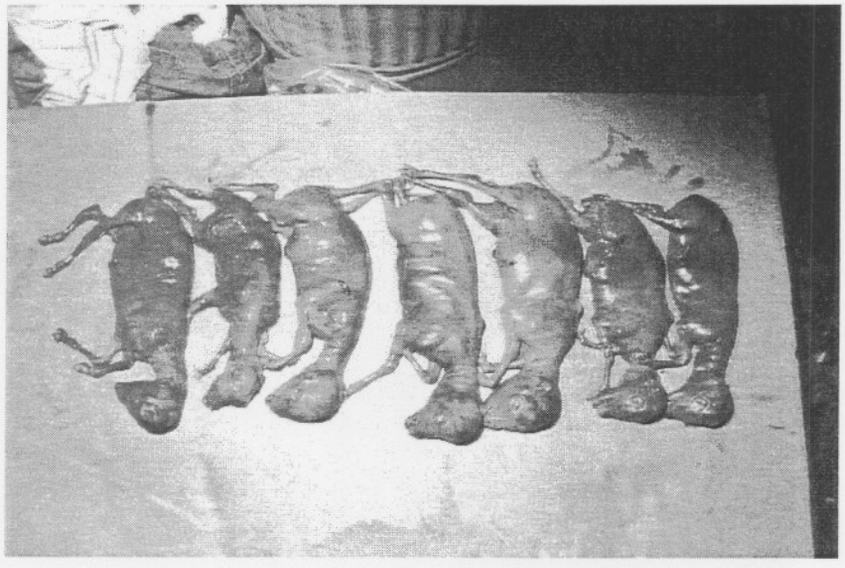
Figure 4: Haemorrhagic state caused in humans by RVFV infection (Paweska, personal communication)

The mortality rate is 1%, but a 15% rate has been observed in hospitalized patients (Gerdes, 2004). RVFV can cross the blood-brain barrier and infect neurons and glial cells, which is why RVFV is known to sometimes cause retinitis and meningoencephalitis in humans (Ritter et al., 2000).

1.4 Effects on animals

Sheep are primarily infected by RVFV but other ruminants including cattle, goats, camels, buffalo and antelope are also susceptible to infection. Young lambs show a fatality rate of 90-100% which is attributed to hepatic liquefaction. Adult sheep have a fatality rate of 20-30% and up to 90% of pregnant ewes abort after being infected. This can be attributed to very high levels of viremia, which also increases the chances of transmission to humans handling infected sheep (Woods et al., 2002). Infected hepatocytes are probably the major source of high plasma viremia observed in infected animals (Ritter *et al.,* 2000). In non-pregnant adult animals clinical signs include listlessness, abdominal pain, vomiting, diarrhoea, jaundice hepatitis, icterus, nasal discharge and death in some cases. Onset of abortions and high neonatal mortality are the characteristics of these outbreaks. Animals like buffalo and camels do not exhibit disease but they do abort when infected (Gerdes, 2004). The experimental incubation period in young animals is 12 hours and 3 days in adult animals (Mebus, 1998).

Figure 5: Abortions in sheep caused by RVFV infection (Paweska, personal communication)



1.5 Diagnostics

Diagnosis of RVFV antibodies can be achieved through serological tests in combination with clinical observations. Detection of antibodies against RVFV includes tests such as haemagglutination-inhibition, indirect immunofluorescence, virus neutralization and complement fixation (Paweska et al., 2005b). The use of these methods is limited to endemic areas, because live virus is used in these assays. The live virus antigen is a serious health risks for laboratory personnel conducting these tests. Virus neutralization is regarded as the gold standard but it is laborious, expensive and requires 5-7 days for completion. It can only be performed when standardized stock of live virus and tissue culture are available (Paweska et al., 2005b).

Rapid detection and identification of RVFV is vital in areas faced with an epidemic. Therefore, various efforts have been made to develop nucleic acid based techniques for this purpose, since the traditional virus isolation and identification methods take a relatively long time for completion and are laborious (Paweska et al., 2005b). Molecular diagnostic tools utilize the direct detection of either the virus RNA or protein components. Several reports have already been published on the use of reverse-transcription PCR (RT-PCR) for detection of RVFV (Garcia et al., 2001; Sall et al., 2002; Sall et al., 2001). This method is used to detect the presence of viral genomic material in clinical samples, and is highly sensitive and reproducible (Zaki et al., 2005). Viral nucleic acid will only be present in the sample if it was collected while the virus was still present in the particular patient or animal. However, once the virus has been cleared by the body, this method is no longer adequate for viral detection. It is, therefore, inadequate to use RT-PCR alone to determine if animals were exposed to the virus, especially because of short viraemia during RVF infection.

Another diagnostic approach is to detect antibodies against RVFV in patients and animals. Experimental inoculation of humans with a killed vaccine resulted in an early IgM response which becomes undetectable by 4 to 6 weeks. In natural infections that were followed closely, IgM antibodies appeared around day 5, were absent in 50% by week 6 and undetectable at 4 months. IgG appears at day 4 and may persist indefinitely. This means that the presence of the immunoglobulin-M (IgM) antibody against RVFV antigen indicates a recent (4-6 weeks) exposure to RVFV. Since IgG against RVFV antigen persists indefinitely in the infected individual, its presence could indicate a less recent infection (Woods et al., 2002). The presence of these antibodies can be measured by different ELISA methods. The effectiveness of various ELISA methods for the detection of antibodies against Rift Valley fever virus has been reported: IgM capture ELISA for the detection of IgM; and IgG sandwich and IgG indirect ELISA for the detection of IgG. An inhibition ELISA also exists for the detection of antibodies against RVFV. ELISA is a better alternative to other detection methods because of its safety, precision and sensitivity. It is also less expensive than other tests and can be easily automated for large scale testing. This test can also be conducted outside of endemic areas because inactivated virus is used (Niklasson et al., 1984; Paweska et al., 2003a; Paweska, Burt, and Swanepoel, 2005; Paweska et al., 2005b; Paweska et al., 2003b). Cross-reaction has been found between RVFV and other Phleboviruses when haemagglutination inhibition and immunofluorescence is used for detection of antibodies. Niklasson *et. al.*, (1984) found that the ELISA based on a betapropiolactione-inactivated virus whole antigen, is able to distinguish between these different viruses. Sera with IgG antibodies against other Phleboviruses tested negative in their ELISA (Niklasson *et. al.*, 1984). Other advantages of using ELISA for the detection antibodies are that they are less expensive and time-consuming can be produced in the form of kits, well standardized and quality controlled. However, the traditional methods of producing and purifying whole viral antigens are expensive and laborious because it is dependent on the ability of the specific virus to multiply in the cell culture. It has also been found that such antigens do not easily coat plates, and therefore cannot be used for indirect ELISA (Paweska, Burt and Swanepoel, 2005).

In theory, natural proteins are the ideal antigens for use in diagnostic assays because they provide sequence-specific epitopes in their natural conformation. However, these antigens have to be produced from cell-culture or isolated from an infected animal. These methods of preparation are

expensive and time-consuming, and each new batch requires standardization. Recombinant DNA technology has the ability to produce antigens that are more pure and of a high specificity. The batch to batch production of these antigens is also more reproducible. Production of recombinant nucleoprotein has been accomplished previously, but these proteins were not used as antigens in ELISA. In one case the recombinant was produced by using the Semliki Forest virus as a vector for expression in mosquito cells. This protein was used to examine the effect thereof on the replication of the virus in mosquito cells (Billecocq et al., 2000). It was also used for the identification of monoclonal antibodies against RVFV N in a preperation (Zaki et al., 2005).

A rapid technique for the detection of IgM antibodies against RVFV has been investigated previously (Soliman *et. al.*, 1988). This method is a solid-phase immunosorbent technique based on haemagglutination inhibition using pre-coated plates. This test was reliable and had sensitivity and specificity comparable to ELISA, results are obtained in five hours and the reagents are inexpensive. However, it was also based on the use of a beta-propiolactone-inactivated virus as antigen (Soliman *et. al.*, 1988).

1.6 Problem formulation and objectives of this study

From a review of the literature as well as the feedback from the practising medical and veterinary health community it is clear, there is an increasing demand for high-quality, cheap and safe serological tests for the diagnosis of RVFV in order to ensure prevention of infection in man and the safe international movement of domestic animals. Thus far, only inactivated virus has been used as antigen for detection with ELISA. Since RVFV is a zoonotic pathogen the current diagnostic antigen needs to be prepared in high containment facilities and by immunized people. There is thus a need for a recombinant antigen (rAg) which would lower costs and safety risks, and enable preparation of antigen and detection of the virus in routine diagnostic laboratories.

The aims of this project were to:

- clone and sequence a gene encoding a RVFV nucleoprotein (Chapter 2)
- express the cloned N-gene in insect cells using the baculovirus system (Chapter 3) and bacterial (Chapter 4) expression systems
- purify a recombinant RVFV N protein (Chapter 4)
- evaluate the suitability of purified recombinant RVFV N protein as a diagnostic antigen in ELISAs (Chapter 5)

1.7 Control and prevention

Surveillance of RVFV can be achieved by routine screening of abortions, sentinel herd monitoring and possibly through the use of satellite weather data. The 1987 RVFV outbreak in Mauritania was predicted five months in advance based on rainfall patterns and vegetation indexes (Gerdes, 2004). A

person can prevent infection with RVFV by taking certain measures to avoid biting by mosquitoes and other potential bloodsucking carriers of RVFV. This can be done through the use of mosquito repellants and bed nets. Farm workers should also avoid contact with blood or tissues of animals that may potentially be infected, especially in RVFV endemic areas (Anonymous, 2004). Control measures for RVFV include movement control of livestock, vector control and vaccination of livestock. The latter is important to stop the virus from being amplified in the livestock and decrease the risk of young/newborn to become infected (Gerdes, 2004).

In RVFV enzootic areas vaccination is the only practical method of preventing the disease. Two types of vaccines have been developed for RVFV: inactivated and live-attenuated. An experimental formalin inactivated RVF vaccine has been used to immunize animals, laboratory workers and other people at risk of exposure to RVFV (Wallace and Viljoen, 2005). The cost of this vaccine and the requirement for multiple vaccinations limit its use. This human vaccine has recently been discontinued. Two live-attenuated vaccines exist, one based on the live attenuated Smithburn strain (also known as Smithburn neurotropic strain, SNS) and the other on the MP12 strain modified by induced mutations (Sall et al., 1998). However, the Smithburn strain is pathogenic for humans and causes abortion when administered to pregnant ewes (Mebus, 1998). The MP12 vaccine has been proven to be safe and immunogenic in humans. It is, however, teratogenic when administered to ewes in their first trimester of pregnancy (Sall et al., 1998). Clone 13 is a naturally attenuated strain with a large deletion in the S segment. It is not pathogenic and highly immunogenic (Bouloy et al., 2001). Results in animals (Niklasson *et. al.*, 1983) suggest that the antiviral drug Ribavirin might be useful for treatment of infected individuals.

Humans may be spared the most devastating impact of RVFV by monitoring and controlling infection in animals through vaccination. However, as described previously the current veterinary vaccines still have negative effects on the animals. The critical neutralizing epitopes of RVFV are highly conserved, which means that a single viral strain can protect against all strains (Pittman et al., 1999).

More recently, an inactivated preparation of RVFV has been developed, but is still under Investigation of New Drug status. However, only 3000 doses of the vaccine are available and production of big batches is problematic (Spik et al., 2005). The possibility of developing a DNA vaccine based on the M genome segment of RVFV was exploited. One of the constructs was found to be highly immunogenic in mice, and elicited protective immunity (Spik et al., 2005).

CHAPTER 2

CLONING AND SEQUENCE ANALYSIS OF THE GENE ENCODING THE NUCLEOPROTEIN OF RVFV ZIM688/78

2.1 Introduction

The genome of RVFV consists of three single stranded negative sense RNA segments. The large (L) segment encodes for the viral RNA dependant RNA polymerase. The medium (M) segment encodes for the glycoproteins G1 and G2 projecting through the virus envelope, and non-structural proteins. The small (S) segment encodes for the nucleoprotein (N) and a non-structural (NSs) protein (Battles and Dalrymple, 1988). The S segment utilizes an ambisense coding strategy, with the genes for N and NSs proteins in opposite orientations in the segment (Sall *et al.*1999). Various isolates of the virus have been studied and the sequences of their genomes determined. It was decided to use a strain isolated from a bovid in Zimbabwe in 1978 (RVFV Zim 688/78) of which the sequence has not yet been published.

2.2 Materials and Methods

2.2.1 Virus propagation:

Since RVFV is zoonotic, safety precautions require that all people coming in contact with the virus be immunized and/or that it has to be propagated in containment facilities. Therefore, cell culture work was done at the high containment BSL-3 facility at the Special Pathogens Unit of the National Institute for Communicable Diseases by Dr. J.T. Paweska, who is immunized against RVFV. Five 75 cm^2 flasks with BHK-21 cells confluent monolayers (8×10^6 cells per flask) were inoculated with the Zim688/78 RVFV virus strain isolated from a bovine that died during the 1978 Zimbabwe epidemic near Harare. The titre of the stock virus was 10^7 $TCID_{50}$/ml. BHK-21 cell were infected at the multiplicity of infection (MOI) of 1×10^{-3} $TCID_{50}$. The virus was harvested after 3 days and stored at 4°C for RNA extraction.

2.2.2 Enzyme-linked-immunosorbent assay:

To confirm the presence of RVFV antigen in tissue culture supernatant and cell suspension flasks an antigen capture ELISA was done by Dr. J.T. Paweska as follows:

1. The wells of a 96 well plate were coated overnight either with 100 µl sheep anti-RVFV antibody (a 1:400 dilution) or RVFV negative sheep serum for baseline control as indicated under Results.

2. Non-specific binding sites were blocked with 200 µl 10% milk powder for 1 hour at 37°C in a humidity incubator.
3. Test samples and controls (100 µl) were added as described under Results and incubated for 1 hour at 37°C in a humidity incubator.
4. Mouse anti-RVFV antibody from NICD stock (100 µl) was added at a dilution of 1:1000.
5. Goat anti-Mouse IgG conjugated with horseradish peroxidase (Zymed, catalogue number 62-6520) was added at a dilution of 1:2000.
6. Horseradish peroxidase substrate, 100 µl ABTS (KPL, catalogue number 50-66-01), was added to the wells and the plate incubated at room temperature in the dark for 30 minutes.
7. The reaction was stopped with 1% SDS.
8. The optical density (OD) values were recorded using a Universal plate reader (EL_X 800, BIO-TEK Instruments) at a wavelength of 405nm.

2.2.3 Viral RNA extraction:

Viral RNA was isolated by using two different commercially available kits:

i) QIAamp® Viral RNA Mini Spin Protocol (QIAgen, Catalogue number 51104)

This kit was used for RNA isolation from cell culture supernatant according to the instructions of the manufacturer, with minor modifications as indicated below. This kit combines the selective binding properties of a silica-gel-based membrane with the speed of microspin technology. The sample is first lysed under highly denaturing conditions to inactivate RNases to ensure isolation of intact viral RNA. The lysis buffer contains carrier RNA which improves the binding of the viral RNA to the QIAamp membrane. After lysis the RVFV is non-infective. The RNA is then bound to the QIAamp membrane during two centrifugation steps. The buffers used in the binding steps provide the optimal binding conditions for RNA, but ensure that protein and other contaminants are not retained on the membrane. The contaminants are then washed away by using two different wash buffers and two short centrifugation steps. The RNA is then eluted in an RNase-free buffer that contains 0.04% sodium azide to prevent microbial growth and contamination with RNases.

The following modifications were made:

- The lysis steps were done by Dr. J.T. Paweska because RVFV is still infectious before lysis.
- The centrifugation in the wash step with Buffer AW2 was done at 10 000 × g for 5 minutes instead of at 20 000 × g for 3 minutes because of the rotation limit of the table centrifuge used.

Viral RNA was resuspended in 60 µl Buffer AVE (RNase-free water containing 0.04% sodium azide). The yield of viral RNA isolated from biological samples is usually less than 1 µg.

ii) TRIzol® Reagent (Invitrogen, Catalogue number 15596-018)

This kit was used for RNA isolation from homogenized cells from the cell culture monolayer in the culture dish according to the instructions of the manufacturers with slight modifications. TRIZol reagent is effective for the isolation of total RNA from cells and tissues. The reagent is a solution of phenol and guanidine isothiocyanate. During the homogenization or lysis of the sample, the TRIzol Reagent serves to maintain the integrity of the RNA while disrupting cells and dissolving the cell components. The addition of chloroform followed by centrifugation accomplishes a phase separation, creating an aqueous and organic phase. The RNA is present in the aqueous phase, and is then precipitated with isopropyl alcohol for recovery.

The following modifications were made:

- Homogenization and phase separation was done by Dr. J.T. Paweska because RVFV is still infective at these stages
- The RNA pellets were redissolved in 100 µl Buffer AVE (RNase-free water containing 0.04% sodium azide) from the QIAamp kit (QIAgen, Catalogue number 51104) for storage instead of RNase-free water or SDS. This method yields approximately 4 µg RNA from 1×10^6 cultured BHK-21 cells.
- The samples were not incubated at 55 to 60°C for 10 minutes (the RNA pellet dissolves sufficiently without this step)

2.2.4 One step Reverse Transcriptase PCR:

The following one step RT-PCR methods were executed:

The Titan™ One Tube RT-PCR System from Roche (Catalogue number 1855476), which contains AMV-RT and Expand™ high fidelity enzymes, was used for diagnostic purposes. The reaction mixtures were set up as follows: 10 µl RNA, 10 µl 5 × RT-PCR Buffer (7.5 mM $MgCl_2$ and DMSO), 1 µl enzyme mix, 0.2 mM dNTPs, 5 mM DTT, 10U of RNasin and 12 pmoles of each primer to a final volume of 50 µl. The reactions were incubated in a Hybaid Touchdown thermocycler (Hybaid Limited, Middlesex, United Kingdom) at 50°C for 30 minutes followed by 30 cycles of 95°C for 30 seconds denaturation, 47°C for 30 seconds annealing and 68°C for 30 seconds elongation, and one final elongation cycle at 68°C for 10 minutes. The samples were held at 4°C.

A one step RT-PCR method, adapted from a RVFV diagnostic method used at the Special Pathogens Unit of the NICD and a method used by Dr. A.C. Potgieter and Prof. A.A. van Dijk on equine encephalosis virus (personal communication) was used for N gene amplification. The RT-PCR reaction mixtures were as follows:

i) Denaturing mixtures with a final volume of 12 µl consisted of 10 µl vRNA template from the QIAamp RNA extract and 2 µl of the appropriate primer sets (2 pmol/µl each). Positive control reactions and amplification reactions contained the appropriate primer sets. Sterilized 18Ω H_2O replaced vRNA template in negative control reactions.

ii) The Master mix consisted of 1× Takara Ex Taq Buffer (Takara, catalogue number KA1201AA) (20 mM Mg^{2+}), 10U of AMV Reverse Transcriptase (Promega, catalogue number M5108), 4U of Takara Ex Taq polymerase (Takara, catalogue number KA1201AA), 1.6 mM dNTP mixture (Takara, catalogue number KA1201AA) and 10U of RNasin (Promega, catalogue number N2511). Water was added to a final volume of 38 µl.

The denaturing mixtures were incubated at 65°C for 2 minutes then quickly transferred to an ice bath. Master mix was added and the final RT-PCR reaction mixtures of 50 µl were incubated in an Eppendorf Mastercycler® ep at 42°C for 20 minutes followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 60 seconds, and one final elongation cycle at 72°C for 10 minutes.

The primers used in the one step RT-PCR reactions are set out in table 1:

Table 1

Primer	Sequence	Length	Tm
009 Forward (Complementary to vRNA)	5' – CCAAATgACTACCAgTCAgC – 3'	20mer	60°C
007 Reverse (Viral sense)	5'- gACAAATgAgTCTggTAgCA – 3'	20mer	58°C
RVFVNPN N-terminal HindIII	5'-AATT**AAgCTT**gATACAAACACTATTACAATAATg–3'	34 mer	60.7°C
RVFVNPN3 N-terminal BamHI	5'–AATT**ggATCC**gATACAAACACTATTACAATAATg–3'	34 mer	62.2°C
RVFVNPC C-terminal XhoI	5' – AATT**CTCgAg**CCCCTgggCAgCCAC – 3'	25 mer	72.3°C

The diagnostic primers, 009 forward and 007 reverse were adapted from the literature (Battles and Dalrymple, 1988) and obtained from the Special Pathogens Unit, NICD.The primers RVFVNPN, RVFVNPN3 and RVFVNPC were designed using the sequence of the MM12 strain as reference, and synthesized and HPLC purified by the company Metabion (www.metabion.com).

2.2.5 PCR Clean-up:

For direct cloning amplicon purification was done using the Promega Wizard® SV Gel and PCR Clean-Up System (Promega, Catalogue number A9281), according to the instructions of the manufacturer. This system is based on the ability of DNA to selectively bind to a silica membrane in the presence of chaotropic salts. Contaminants like excess nucleotides, primers, enzymes and salts are removed by this system, while purified DNA is eluted in 50 µl nuclease-free water.

2.2.6 Agarose gel electrophoresis:

Agarose gel electrophoresis was done as set out in the literature (Sambrook and Russell, 2001). 1.0% gels (6 cm × 10 cm × 0.5 cm) were prepared in TAE (Tris-Acetate-EDTA) buffer containing ethidium bromide (0.5 µg/ml) (Sambrook and Russell, 2001). All gels were prepared with TAE buffer unless otherwise indicated. Other gels were prepared with TBE buffer (Sambrook and Russell, 2001). O'GeneRuler DNA ladder mix (Fermentas, Catalogue number SM1173) was used as a DNA size marker. Two different loading buffers were used: 6X Orange loading dye solution containing two dyes, Orange G and xylene cyanol FF (Fermentas, Catalogue number R0631) and 5X Gel loading buffer (25% bromophenol blue, 25% xylene cyanol FF and 30% glycerol in H_2O)(Sambrook and Russell, 2001) Gel electrophoresis was performed using a Bio-Rad PowerPac Basic system at 100V. DNA was visualized with UV transillumination. DNA concentration was measured at an OD of 260 nm using a NANODROP spectrophotometer (NanoDrop® ND-1000 Spectrophotometer). DNA purity was measured using the ratio of 260/280 nm also using the NANODROP system. Agarose gels were visualized using a Gel-documentation system (Syngene ChemiGenius Bio-Imaging System) and GeneSnap software (Syngene, England).

2.2.7 Gel-extraction:

Gel electrophoresis using a 1.0% agarose gel in TAE buffer was performed (Sambrook and Russell, 2001) with slight modifications. Preparative gel wells of 1.4 cm long were made by taping two 0.5 cm wells together. Loading mixtures had a total volume of 60 µl, which consisted of 50 µl DNA sample, and 10 µl loading buffer (5X Gel loading buffer)(Sambrook and Russell, 2001). Gels were run until the bromophenol blue migrated approximately 6 cm.

Gel extraction was done using the QIAquick Gel Kit Protocol (QIAgen catalogue number 28704) according to the instructions of the manufacturer. This kit utilizes the spin-column technology and selective binding properties of a silica-gel membrane. DNA absorbs to the silica-membrane in the presence of high chaotropic salt concentrations and at the correct pH (≤7.5) while contaminants pass through the column. The impurities (primers, salts, enzymes, unincorporated dNTPs, agarose, ethidium bromide) are washed away with wash buffers and the pure DNA is eluted with a Tris buffer.

2.2.8 A/T cloning of RT-PCR amplicon:

Thermostable polymerases, used in PCR reactions, often add single adenine residues to the 3' ends of amplified fragments. These fragments can be cloned into linearized vectors with single 3' thymidine overhangs. This method of cloning is called A/T cloning. A pGEM-T Easy Vector System II (Promega, Catalogue number A1380) was used for A/T cloning of the RVFV N gene. The pGEM-T Easy vector is linearized at base 60 with EcoR V and has single thymidines added to both 3' ends. These 3'-T overhangs enable easy ligation of PCR products with single 3' adenine overhangs into the vector.

Ligation is carried out by T4 DNA ligase. Ligation of the RVFV N gene into pGEM-T Easy was done according to the instructions of the manufacturer. High-efficiency chemically competent JM109 cells, supplied as part of the kit by the manufacturer, were transformed with the ligation reactions using the heat-shock method. The transformation reactions were carried out according to the instructions of the manufacturer. Blue-white colony selection was done according to the instructions of the manufacturer.

The principle of blue white colony selection is as follows. Successful cloning of an insert into pGEM-T Easy interrupts the *lacZ* gene (more specifically the α-peptide of the β-galactosidase coding sequence). B-galactosidase (consisting of an α- and Ω-peptide) normally metabolizes galactose to produce lactose and glucose, but can also convert a colourless substrate such as X-gal (5-bromo-4-chloro-3-indolyl-[beta]-D-galactopyranoside) to produce a blue colored product. The *lacZ* gene contains multiple cloning and A/T cloning sites where inserts can be inserted, which will disrupt the gene. When this happens, a functional β-galactosidase can no longer be produced, and, therefore, X-gal can no longer be metabolized to form a blue coloured product. Thus the colonies with inserts successfully cloned into pGEM-T Easy will be white. If the cloning was unsuccessful, or no DNA insert was included in the ligation reaction, the colonies will be blue because of plasmid re-ligation, causing *lacZ* not to be disrupted and β-galactosidase to be produced normally to metabolize X-gal to a blue product. In some cases blue colonies may represent successful cloning. This happens when the insert is a multiple of 3 bases long (including 3'-A overhangs) and does not contain in-frame stop codons, and therefore does not disrupt the *lacZ* reading frame. When white colonies are observed from background control reactions (that contain no DNA insert), this could be attributed to re-ligation of damaged vector ends. A third type of colony, which is blue in the middle and white on the edges, can sometimes be observed. They are commonly called "bulls-eye" colonies. Possibly a small amount of α-peptide is produced in these recombinants by means of ribosomal frameshifting, second site translational initiation, or as an α-peptide fusion protein. This small amount of α-peptide can cause the initial development of blue color.

2.2.9 Preparation of chemically competent cells:

Chemically competent cells were prepared according to the method described by Inoue (Inoue, Nojima and Okayama, 1990). All glassware used were washed with chromic acid, rinsed several times with distilled water and sterilized by autoclaving. A total of 12 colonies from overnight plates were picked and inoculated in 125 ml LB-broth (containing the appropriate antibiotics) in a 1 litre conical glass flask. These cultures were grown at 18°C with 215 rpm shaking until the culture reached an absorbance at 600nm of 0.6. The flask was placed on ice for 10 minutes. The cells were then collected by centrifugation at 2500g in a Beckman ultracentrifuge. The pellet was carefully resuspended in 40 ml ice-cold transformation buffer (10 mM PIPES, 15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$) and placed on ice for a further 10 minutes. The transformation buffer is prepared by adding all the reagents except the $MnCl_2$, adjusting the pH to 6.7 and sterilization by autoclaving. $MnCl_2$ is then added by filtration through a 0.22 µm filter. The cells were collected by centrifugation at 2500 g for 10 minutes at 4°C. The cells were resuspended in 10 ml ice-cold transformation buffer, and

DMSO added to a final concentration of 7%. The mixture was kept on ice for 10 minutes, aliquoted into cryotubes and frozen in liquid nitrogen.

2.2.10 Ligation reactions:

Ligation reactions were carried out as described in the literature (Sambrook and Russell, 2001). Ligation reaction mixtures consisted of 2 × ligation buffer (400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5mM ATP, pH7.8), T4 DNA ligase (2.5 U)(Fermentas, Catalogue number EL0012), specific amounts (see protocols for cloning into the different vectors) of the appropriate vector DNA with BamHI/XhoI cloning ends, specific amounts (see protocols for cloning into the different vectors) of gel-extracted RVFV N gene insert with BamHI/XhoI cloning ends and sterilized H_2O to a final volume of 11 µl. Ligation reactions (lacking the enzyme) were first incubated at 65°C for 60s and quickly transferred to ice, the enzyme was added and the reactions incubated overnight at 18°C.

2.2.11 Transformation of chemically competent cells:

When cells are made competent, their membrane properties are modified to facilitate the uptake of DNA plasmid during the heat-shock step. This method uses the heat-shock technique for transformation of the competent cells with the plasmid. A 50-100 µl volume of chemically competent cells is added to specific amounts of the plasmid DNA (as indicated under Results for each individual cloning experiment), gently mixed and placed on ice for 20 minutes. The cells are then heat-shocked for 50 seconds in a water bath at 42°C, and immediately transferred to ice for 2 minutes. A volume of 900-950 µl SOC or LB medium at room temperature is then added to the tubes and incubated for 1 hour at 37°C while shaking at 150-250 rpm. The transformation cultures (100 – 200 µl) are then spread out on LB-agar plates containing the appropriate antibiotics and where applicable, IPTG and X-gal.

2.2.12 Plasmid purification:

Two commercially available plasmid purification kits were used:

i) QIAprep Spin Miniprep Kit (QIAgen, Catalogue number 27106)

The QIAprep Spin Miniprep Kit generally yields up to 20 µg of plasmid from a 1.5 ml overnight culture. Plasmid purification was done according to the instructions of the manufacturer. This kit uses silica membrane technology and eliminates the use of phenol or ethanol for extraction. The procedure is based on alkaline lysis of the bacterial cells followed by the absorption of DNA onto silica in the presence of a high salt concentration. The procedure consists of three steps: i) Preparation and clearing of the bacterial lysate, ii) adsorption of DNA onto the QIAprep membrane and iii) washing and elution of the plasmid DNA. In the lysis step the bacteria are lysed in a buffer containing NaOH/SDS and RNase A. The SDS solubilizes the phospholipids and protein components in the cell membrane which leads to lysis and release of cell contents. The alkaline conditions denature the chromosomal DNA and proteins. The lysis time is optimized to ensure the optimum release of plasmid DNA without

the release of chromosomal DNA and without exposing the plasmid DNA to denaturing conditions for too long. The lysate is then neutralized and adjusted to a high-salt concentration which causes denatured components to precipitate while only the small plasmid DNA renatures and stays in solution. The plasmid DNA then binds to the membrane of the spin column and is washed free of endonucleases and salts by the wash buffers. The plasmid DNA is then eluted under the correct pH conditions using a Tris Buffer containing low-salt. Plasmid DNA was resuspended in 50 µl Buffer EB (10 mM Tris-Cl, pH 8.5).

ii) PeQLab E.Z.N.A.® Plasmid Miniprep Kit 1 (PeQLab, Catalogue number 12-6943-00)

The PeQLab Plasmid Miniprep Kit generally yields up to 25 µg of plasmid from a 5 ml overnight culture. This kit combines the technology of DNA binding to a membrane and alkaline-SDS lysis of bacterial cells. The plasmid DNA from lysed cells is bound to the membrane in optimal conditions that allow proteins and other contaminant to be removed. After washing the DNA is eluted using a low-salt buffer. Plasmid purifications were done according to the instructions of the manufacturer.

2.2.13 Restriction enzyme digestions:

Restriction enzyme digestions for restriction site analysis were generally carried out in 25 µl reaction mixtures for 2-3 hours. Restriction enzyme double digestions for excision of specific DNA fragments, or preparation of overhanging cloning sites, were carried out in 100 µl reaction mixtures for 5-6 hours. Reaction mixtures consisted of DNA, appropriate 1× restriction enzyme buffer, appropriate restriction enzyme(s) and sterile 18 Ω H_2O. The following restriction enzymes and buffers, obtained from the companies Fermentas and Promega, were used in this project:

BamHI (Fermentas, Catalogue number ER0051); XhoI (Fermentas, Catalogue number ER0691); NdeI (Fermentas, Catalogue number ER0582); SalI (Fermentas, Catalogue number ER0641), NcoI (Fermentas, Catalogue number ER0572), HindIII (Promega, catalogue number R6041), 10× buffer BamHI (10 mM Tris-HCl pH8.0, 5 mM $MgCl_2$, 100 mM KCl, 1 mM 2-mercaptoethanol, 0.02%Triton X-100; 0.1 mg/ml BSA) (Fermentas, Catalogue number B57); 10× buffer Tango (33 mM Tris-acetate pH7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA) (Fermentas, Catalogue number); 10× buffer R (10 mM Tris-HCl pH8.5, 10 mM $MgCl_2$, 100 mM KCl, 0.1 mg/ml BSA) (Fermentas), Buffer E (60 mM Tris-HCl pH7.5, 1 M NaCl, 60 mM $MgCl_2$ and 10 mM DTT) (Promega, Catalogue number R6041), BSA (Promega, Catalogue number R6041).

Different restriction enzymes function optimally in buffers with differing compositions. Important factors in buffer compositions are pH, type and concentration of monovalent cation (K^+ or Na^+). When double digestions are done a compromise has to be made to choose a single buffer for both enzymes to function in. Double digestion reactions were set up according to the recommendations on the Fermentas website (www.fermentas.com).

2.2.14 DNA Sequencing:

Sequencing was done at the commercial laboratories of the Onderstepoort Veterinary Institute (Pretoria, South Africa) and Inqaba Biotech (Pretoria, South Africa). Sequencing reactions done at OVI were performed using the ABI PRISM® Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The sequence reactions were analysed on an ABI PRISM® 3100 Genetic Analyser (*Applied Biosystems*). The sequencing reactions done at Inqaba were performed using the BigDye version 3.1 dye terminator cycle sequencing kit (Applied Biosystems). The sequences were analysed on a Genetic analysis system SCE2410 (SpectruMedix LLC, Pennsylvania, USA) and BaseSpectrum V2.1.1 sofware (SpectruMedix).

Approximately 3-6 ng gel-extracted amplicons (see 2.3.4) were sequenced at Onderstepoort Veterinary Institute using 3 pmol of each of the appropriate primers. Approximately 4 µg plasmid DNA (see 2.3.4) were sequenced at Onderstepoort Veterinary Institute using 3 pmol of each of the appropriate primers.

2.3 Results

2.3.1 RVF virus propagation and viral RNA extraction:

Five confluent 75 cm^2 flasks of BHK21 cells were infected with the RVFV Zim688/78 strain and harvested as described under Materials and Methods (2.2.1). The presence of RVFV was confirmed by an ELISA and a RVFV diagnostic RT-PCR.

Rift Valley fever virus antigen in infected tissue culture supernatant and cell suspension flasks was detected by a RVFV antigen immunocapture ELISA as described under Materials and Methods (2.2.2). The first 48 wells of the 96 well plate were coated with sheep anti-RVFV serum (1:400 dilution) and the other 48 with RVFV negative sheep serum to serve as a baseline control. Samples and controls were added in duplicate to each of the two different coatings. Two strong positive control antigens (Ag++), one weak positive control antigen (Ag+) and one negative control antigen (Ag-) was added from the NICD sample bank. Serial dilutions were made of the cell culture supernatant from Flask1 to 1:200, 1:400, 1:800 and 1:1600, and added in duplicate to the wells. Serial dilutions were made of the cell culture supernatant from Flask2 to 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800 and 1:25600 and added in duplicate to the wells. The cell suspensions of the five 75cm^2 RVFV infected flasks were pooled together and serial dilutions made to 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800 and 1:25600, and added to the wells in duplicate. Mouse anti-RVFV (1:1000 dilution), goat anti-mouse IgG HRPO (1:2000 dilution) and ABTS (HRPO substrate) was then added and the reaction stopped by addition of 1% SDS as described in Materials and Methods (2.2.2). The results obtained can be seen in Figure 6.

From Figure 6 it can be seen that high amounts of RVFV was present in all the samples since the PP value (percentage positivity) of each sample at 1:200 dilution is much higher (PP 140-160%) than that of the positive control samples (PP 100%). There was also no unspecific background since the PP value of the negative control is 0%. Therefore, it was decided that we could progress to the viral RNA extraction.

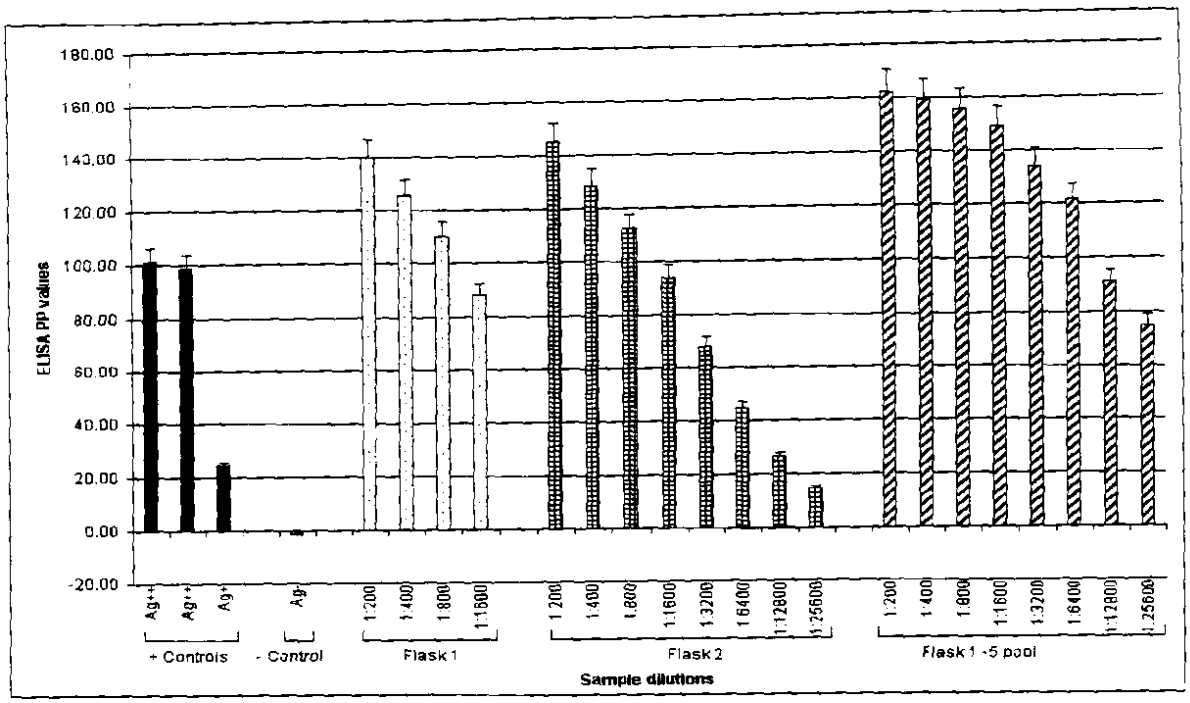


***Figure 6.* ELISA of RVFV present in the infected cells.**

Positive controls (▬) consisted of two strong positive control antigens (Ag++) and one weaker positive control antigen (Ag+). No antigen was added in the negative control mixture (Ag-). The cell culture supernatant from Flask 1 (——) was diluted to 1:200, 1:400, 1:800 and 1:1600. The cell culture supernatant from Flask 2 (▤) was diluted to 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800 and 1:25600. The cell suspensions of Flasks 1-5 (▨) was pooled together and diluted to 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800 and 1:25600.

Viral RNA was extracted by using two different commercial RNA extraction methods as described under MATERIALS AND METHODS (2.2.3). The QIAamp kit was used to extract RNA from cell culture supernatant, where the mature virus is present, to ensure that less cellular RNA is extracted. The TRIzol Reagent was used to extract RNA from homogenized cells, resulting in the extraction of both cellular as well as virus RNA. A one step RT-PCR (MATERIALS AND METHODS, 2.2.4) was performed to validate the presence of viral RNA in the extracted samples (QIAamp and TRIzol extracts). Diagnostic primers that amplify a region of the M-segment of the RVFV genome (a partial sequence of the gene encoding glycoprotein G2) to yield a 369 bp DNA product was used. The negative control reaction mixture contained H_2O instead of RNA while positive RVFV RNA (from the NICD sample bank) was added to the positive control reaction mixture. The diagnostic primers, 009 forward and 007 reverse (Table 1 MATERIALS AND METHODS, 2.2.4) were adapted from the literature (Battles and Dalrymple, 1988).

The PCR products were analyzed by means of gel electrophoresis (Figure 7) using a 1.2% agarose gel in TBE buffer (Sambrook and Russell, 2001). The Titan™ 100bp DNA ladder was used as a DNA marker.

Figure 7 shows that the amplicons of both RNA extraction methods are in the range of 369 bp, and that it aligns with the positive control RVFV amplicon from the gene encoding RVFV glycoprotein G2. This means that enough RVFV RNA was extracted by both extraction methods and that the extracted RNA is indeed RVFV RNA. The lack of a visual band in the H_2O control lane indicates that no contamination of the RT-PCR mixtures with viral RNA took place prior to the PCR reaction. TRIzol RT-PCR amplicon was loaded in wells 2 and 6 because experimental error resulted in only 3 µl of the sample being loaded in well 2, instead of 5 µl.

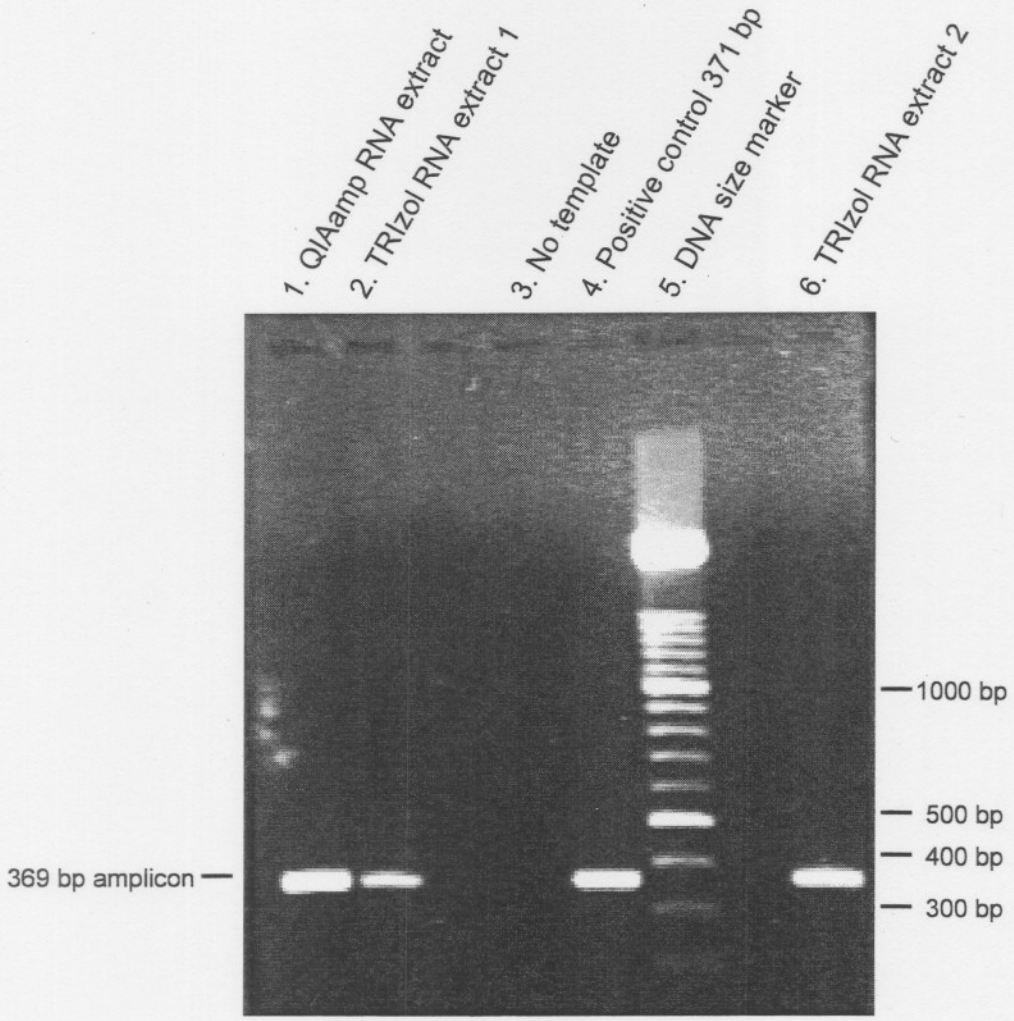


Figure 7. Gel electrophoretic analysis of the amplicons of a diagnostic RVFV RT-PCR performed on viral RNA extracted with two different commercial methods, QIAamp and TRIzol. Lanes: 1) 5 µl amplicon of QIAamp RNA extract; 2) 3 µl amplicon of TRIzol RNA extract; 3) Water control; 4) 5 µl amplicon of positive RVFV RNA control; 5) 100 bp DNA molecular weight marker XIV (Roche, Catalogue number 1721933); 6) 5 µl amplicon of TRIzol RNA extract.

2.3.2 One step RT-PCR for amplification of the gene encoding the N protein of the Zim688/78 RVFV strain:

The gene encoding the RVFV N protein is located in the negative sense on the S segment of RVFV RNA and consists of 738 bases. The forward primer RVFVNPN and the reverse primer RVFVNPC were designed for the amplification of the RVFV Zim688/78 N gene for subsequent A/T cloning into cloning vector pGEM-T Easy. The forward primer RVFVNPN3 was designed and used with the reverse primer RVFVNPC for amplification of the RVFV Zim688/78 N gene for subsequent directional cloning into expression vectors. During the RT-PCR cDNA is generated first and then amplified to yield a 794 bp dsDNA product with 3' adenine overhangs.

One step RT-PCR was performed as described in MATERIALS AND METHODS (2.2.4). Negative control reactions were done without adding any template. Positive control reactions were done using the RVFV diagnostic primers, and RVFV positive RNA, obtained from the SPU of the NICD.

For A/T cloning and direct amplicon sequencing the RVFV N gene was amplified with the RVFVNPN and RVFVNPC primers, and subsequently gel-extracted with the QIAquick Gel Kit Protocol (QIAgen Catalogue number 28704) for purification.
A 5µl volume of each 50 µl RT-PCR mixture was analyzed by gel electrophoresis on a 1.0% agarose gel depicted in Figure 8.

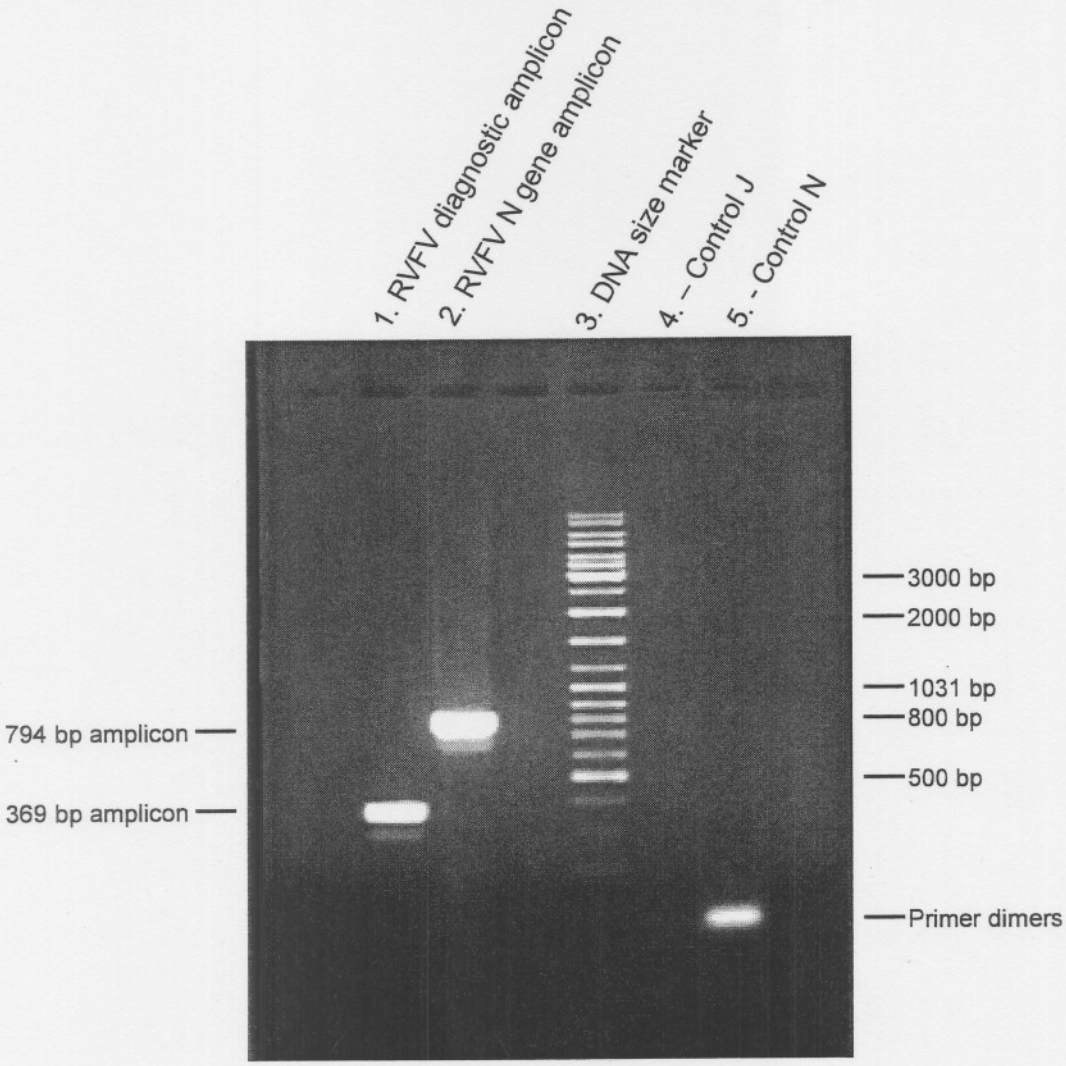


Figure 8. Gel electrophoretic analysis of amplicons for A/T cloning and direct sequencing, of the one step RT-PCR performed on RVFV Zim688/78 QIAamp extracted RNA using RVFV diagnostic primers and N gene primers.

Lanes contain 5 µl of each 50 µl RT-PCR amplicon: 1) Amplicon of RT-PCR using RVFV diagnostic primers; 2) Amplicon of RT-PCR using RVFVNPN and RVFVNPC primers; 3) O' GeneRuler DNA marker (Fermentas, Catalogue number SM1173); 4) Negative control with RVFV diagnostic primers containing no RNA and 5) Negative control with N protein primers containing no RNA.

Figure 8 shows that the positive control RT-PCR reaction in lane 1 resulted in a 369 bp amplicon as expected (compare Diagnostic RT-PCR results in Figure 7). This indicates that the RT-PCR reaction was successful. The amplicon in lane 2 is approximately 800 bp according to the DNA size marker, which is consistent with the expected size of 794 bp of the N gene amplicon. The correct size of the amplicon, the absence of non-specific bands and the intensity of the band in lane 2 means that the gene encoding the N protein of RVFV Zim688/78 was amplified successfully with the RT-PCR. The lack of a visual band in the negative control lane 4 indicates that no RNA contamination took place. The visual band in lane 5 is due to primer dimers, because of the large amount of primers that was used (100 pmoles of each).

The next step was to purify the amplicons by removing RNA, oligonucleotides, proteins and other contaminants by means of gel extraction to ensure good quality DNA for sequencing and cloning. A modified gel electrophoresis and subsequent gel-extraction (Sambrook and Russell, 2001) was done on the remaining amplicons. A 3 µl volume of each gel-extracted amplicon was analyzed by gel electrophoresis on a 1.2% agarose gel depicted in Figure 9.

Figure 9 shows that the gel-extraction of both amplicons was successful. This meant that these gel-extracted amplicons were ready to be used for sequencing and cloning.

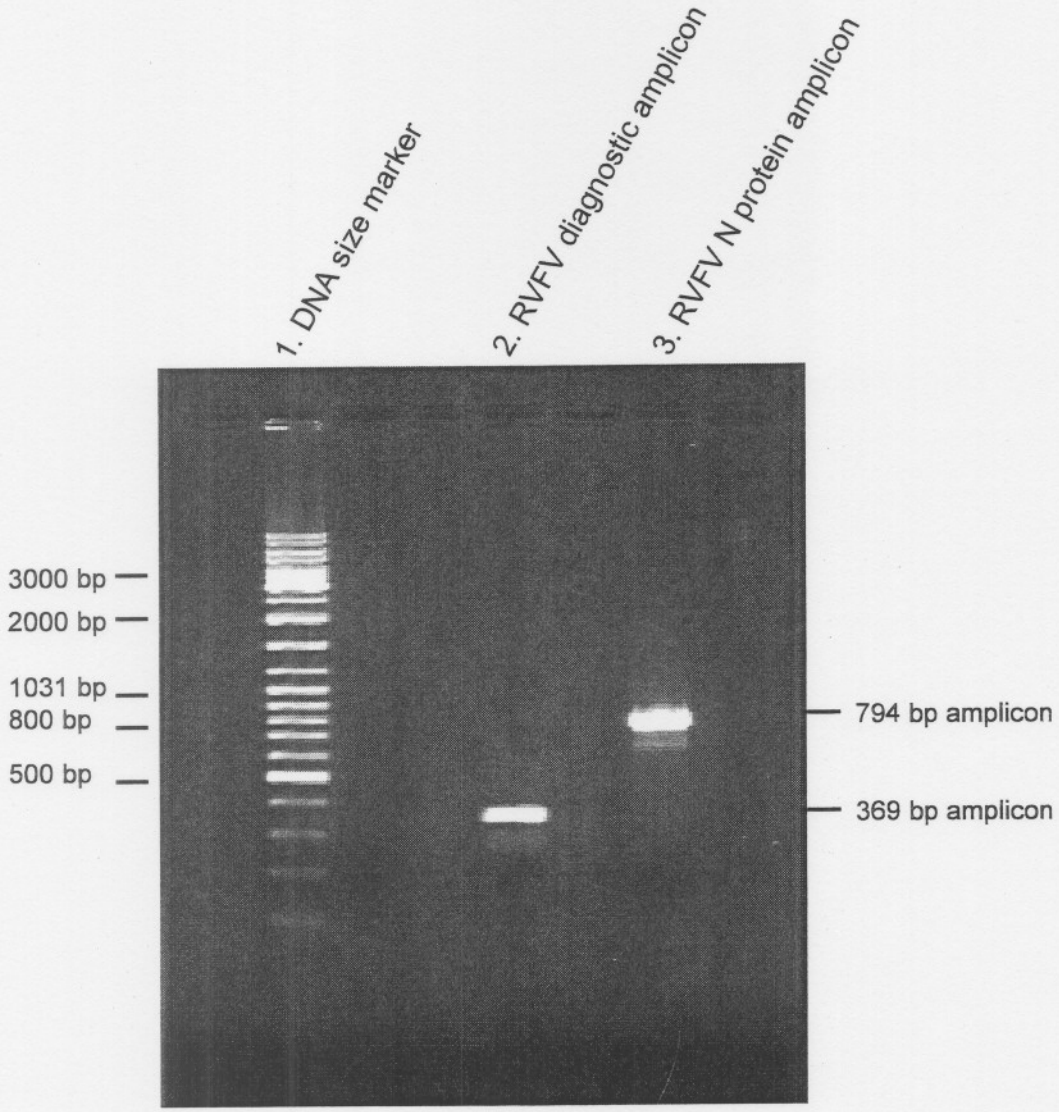


Figure 9. Gel electrophoretic analysis of gel-extracted RVFV Zim688/78 diagnostic and N gene amplicons. Lanes: 1) O' GeneRuler DNA marker (Fermentas, Catalogue number SM1173); 2) 3µl of 30 µl gel-extracted RVFV Zim688/78 diagnostic amplicon and 3) 3µl of 30 µl of gel-extracted RVFV Zim688/78 N gene amplicon.

For directional cloning four identical amplification mixtures, using RVFVNPN3 and RVFVNPC primers, were set up to in order to obtain enough amplicon for further cloning experiments. A 2 µl volume of each 50 µl RT-PCR mixture was analyzed by gel electrophoresis on a 1.0% gel depicted in Figure 10.

The lack of a visual band in lane 2 of Figure 10 indicates that no RNA contamination took place since no vRNA was added in this reaction. The band at the bottom of lane 2 can be attributed to primer dimers due to the large amount of primers that was used (100 pmoles of RVFVNPN3 and RVFVNPC each). The band in the positive control lane 3 at approximately 370 bp indicates that the RT-PCR was successful since the diagnostic primers are expected to yield a 369 bp product. The bands in lanes 4-7 are approximately 800 bp in size, which correlates with the expected N gene amplicon size of 794 bp (including N gene and primers). According to these results an amplicon of the gene encoding the N protein of RVFV Zim688/78 was successfully generated by the one step RT-PCR.

The RT-PCR amplicons were purified using the Promega Wizard® SV Gel and PCR Clean-Up System. A 2 µl volume of each purification was analyzed by gel electrophoresis on a 1.0% gel depicted in Figure 11.

The presence of visual bands in lanes 2-5 of Figure 11 at an approximated size of 794 bp indicates that the purification of the N gene amplicons with the Wizard® SV Gel and PCR Clean-Up System was successful.

The purified N gene RT-PCR products were pooled together and the DNA concentration and purity determined using a NanoDrop® ND-1000 UV-Vis Spectrophotometer. The DNA concentration was determined to be 194.79 ng/µl with purity (A260/280) being 1.86. This indicates a high yield of DNA with good purity. The RVFV N gene amplicons were now ready for further use in directional cloning.

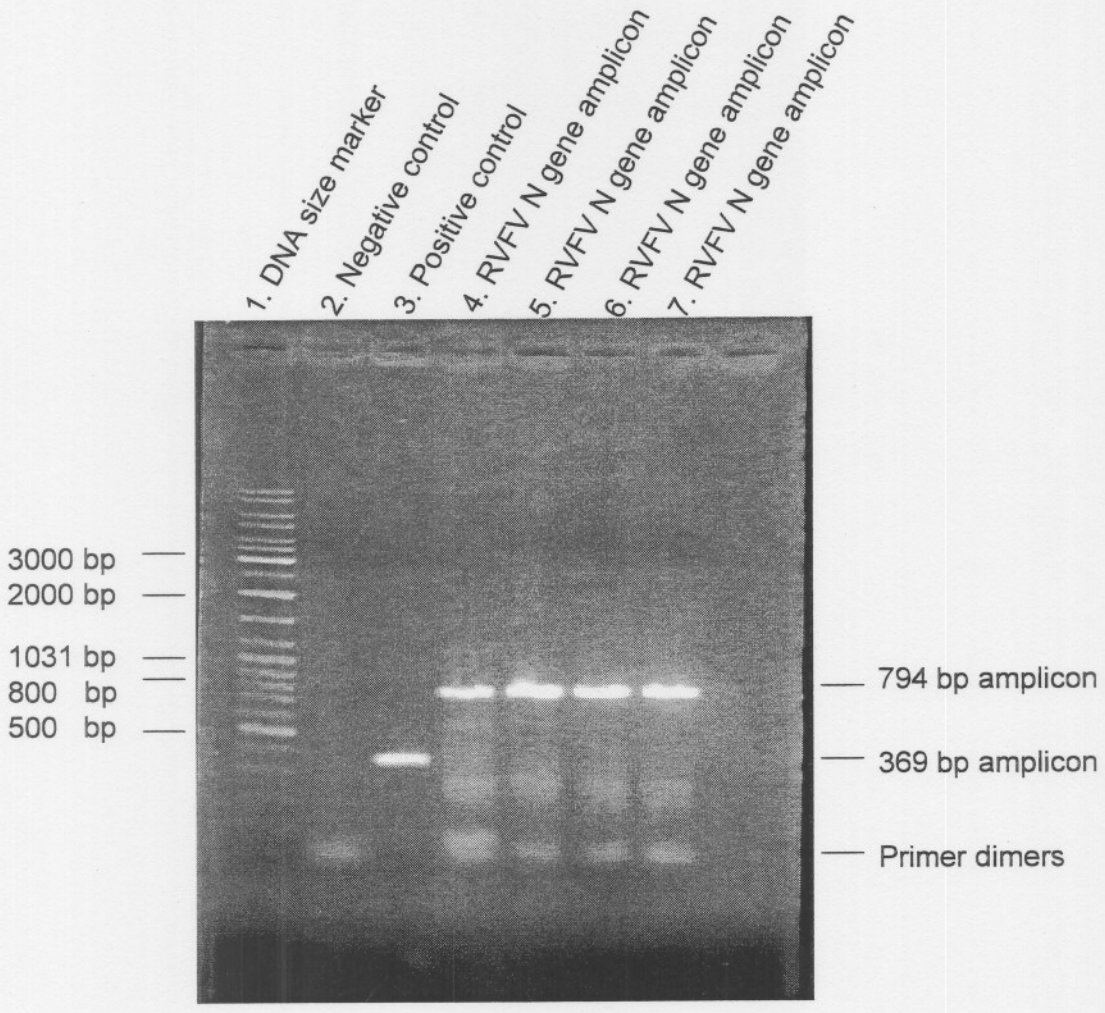


Figure 10. Gel electrophoretic analysis of one step RT-PCR amplicons, for directional cloning, from QIAamp extracted RVFV Zim688/78 RNA using diagnostic primers and N gene primers.

Lanes contain 2 µl of each 50 µl RT-PCR amplicon: 1) O' GeneRuler DNA size marker (Fermentas, Catalogue number SM1173); 2) Negative control containing no vRNA; 3) Amplicon of positive control RT-PCR with diagnostic primers; 4-7) Amplicons of amplification reactions using RVFVNPN3 and RVFVNPC primers.

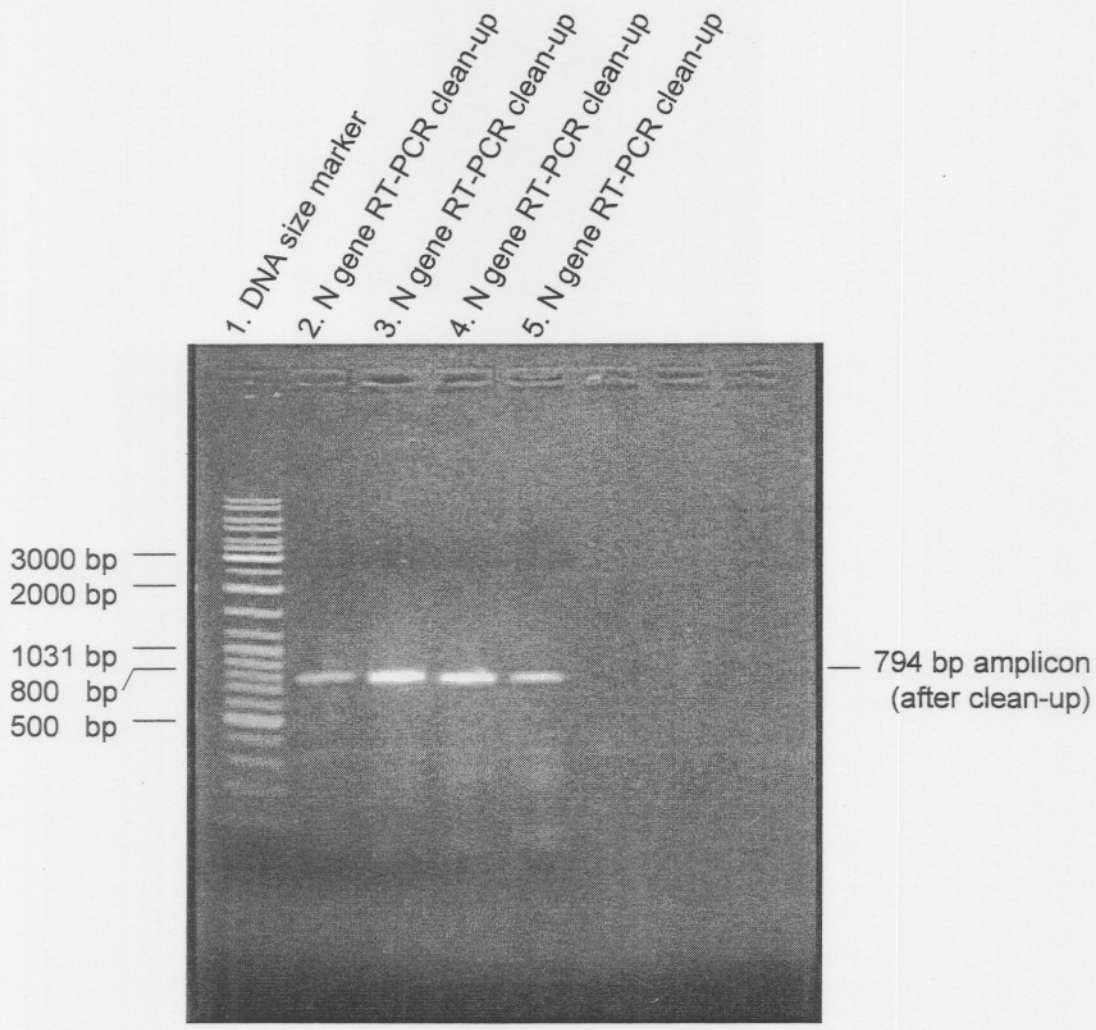


Figure 11. Gel electrophoretic analysis of N gene amplicons from the one step RT-PCR after Promega Wizard® SV Gel and PCR Clean-Up. Lanes contain 2 µl of each 50 µl clean-up reaction: 1) O' GeneRuler DNA size marker (Fermentas, Catalogue number SM1173); 2-5) Clean-up RVFV N gene amplicons.

2.3.3 A/T cloning of the RVFV Zim688/78 N amplicon into pGEM-T Easy:

The commercial cloning vector pGEM-T Easy was chosen for cloning of the gene encoding the N protein of RVFV Zim688/78. It is linearized at base pair 60 and has overhanging thymidines added to the 3' ends to facilitate A/T cloning of amplicons with overhanging 3' adenines. Once the amplicon is cloned into pGEM-T Easy it could be excised by using restriction enzymes that cut in the multiple cloning site of the vector. Specific restriction sites had to be designed and incorporated into the primers to facilitate the subsequent insertion of the cloned amplicon into the multiple cloning site of the various expression vectors.

The RVFV Zim688/78 N protein amplicon was ligated into pGEM-T Easy as described in MATERIALS AND METHODS (2.2.8 and 2.2.10). The positive control reaction contained a control DNA insert provided with the kit (a 542 bp fragment with multiple stop codons) instead of the RVFV N protein amplicon. The background control contained no DNA insert or amplicon. In the reaction mixture 3 µl of the 50 µl RVFV N gene RT-PCR amplicon was used for ligation. Transformation of chemically competent JM109 competent cells, using the abovementioned ligation reaction mixtures, was done as described in MATERIALS AND METHODS (2.2.11). The transformation cultures were plated on LB/amp/IPTG/X-gal plates and incubated overnight.

Blue, white and "bulls-eye" colonies were obtained from the overnight plates. The number of colonies of each colour on five representative plates was counted and the percentage composition of each colour was calculated. These results can be seen in Table 2. White and bulls-eye colonies on LB/amp/IPTG/X-gal plates represent the colonies where cloning of an insert into pGEM-T Easy, and the transformation of competent cells, was successful. Blue colonies represent unsuccessful cloning, since the *lacZ* gene is not disrupted in the plasmids in these cells. According to the results in Table 2 approximately 79.75% of the total colonies from N gene cloning represented successful cloning. This correlates with the 77.68% successful cloning of the positive control (control insert). The negative control plate yielded the expected result as all the colonies were blue.

Table 2. The percentage of each colony colour on representative LB/amp/IPTG/X-gal plates containing transformed cells

	Background control	Positive control	RVFV N gene
Blue	100%	21.43%	20.24 %
White	0%	0.89%	46.88 %
Bulls eye	0%	77.68%	32.87 %

Colonies representing every colour formation were selected, transferred to LB/amp/IPTG/X-gal masterplates and each colony given a number according to their positions on the masterplates as described in Table 3.

Table 3. Numbers and descriptions of selected colonies of the cloning experiment.

No.	Colour	Amplicon used for cloning	No.	Colour	Amplicon used for cloning
A1	White	N prot1	E1	White	N prot1
A2	White	N prot1	E2	Blue	Background control
A3	White	N prot1	E3	Bulls eye	Background control
A4	Blue	N prot1	E4	Bulls eye	Positive control
A5	White	N prot1	E5	White	Positive control
B1	Bulls eye	N prot1	F1	White	N prot1
B2	Bulls eye	N prot1	F2	White	N prot1
B3	White	N prot1	F3	White	N prot1
B4	White	N prot1	F4	White	N prot2
B5	Blue	N prot1	F5	White	N prot2
C1	White	N prot1	G1	Bulls eye	N prot2
C2	White	N prot1	G2	White	N prot2
C3	White	N prot1	G3	White	N prot2
C4	White	N prot1	G4	White	N prot1
C5	White	N prot1	G5	Bulls eye	N prot1

A sample of each colony was propagated for 18h at 37°C with shaking in 10 ml LB-amp cultures. Plasmid purification was done on these cultures as described in MATERIALS AND METHODS (2.2.12). A 5 µl volume of each 50 µl purified plasmid preparation was then analyzed by gel electrophoresis on a 1.2% agarose gel depicted in Figure 12.

The upper half of Figure 12 shows that the plasmids in lanes 1, 2, 4, 6, 8, 9, 10, 11 and 12 align. This is consistent with the expected result because all these plasmids were isolated from white or bulls-eye colonies from Nprot1 plates. These have migrated slower through the gel than those in lanes 3, 5 and 13. This is also consistent with the expected results, since the latter plasmids were all isolated from blue colonies. This means that they have no sequence cloned into them and are thus smaller than the plasmids from the white or bulls-eye colonies (which originated from ligations with the N protein amplicon), explaining their further migration. The lower half of Figure 12 shows that the plasmids in lanes 17, 18, 19 and 21 to 26 align. This is consistent with the expected result because all these plasmids were isolated from white or bulls-eye colonies from N prot1 and N prot2 plates. Thus, these plasmids probably have the N gene amplicon successfully cloned into them. The plasmid in lane 14 has migrated further than the rest of the plasmids in the lower half, which is consistent with the expected result. This plasmid was isolated from a bulls-eye colony from a background control plate and, since no DNA insert was used, it is smaller than the plasmids from white colonies. One possible explanation for the presence of a bulls-eye colony on the background control plate (which should theoretically contain only blue colonies) is that it could be attributed to the loss of the 3'-T overhangs which would also result in the interruption of the

ORF of the *lac*Z gene. The plasmids in lanes 15 and 16 have migrated a little further than the other plasmids from white colonies. This is consistent with the expected results because these plasmids were isolated from white or bulls-eye colonies from a positive control plate. The control DNA insert used in the positive control reactions is a 542 bp fragment, which is 252 bp smaller than the N protein amplicon. Therefore, the cloned plasmids containing this insert will migrate a little further than those containing the N protein amplicon. These results were encouraging, but restriction analysis and sequencing was still necessary to confirm the successful cloning.

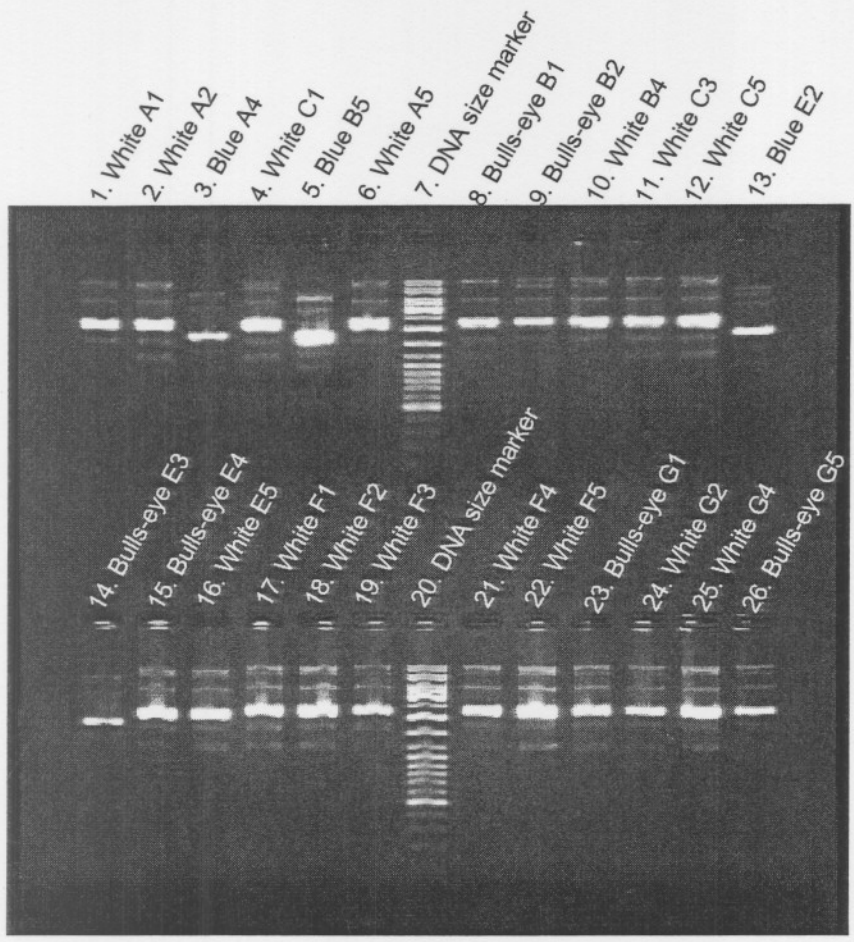
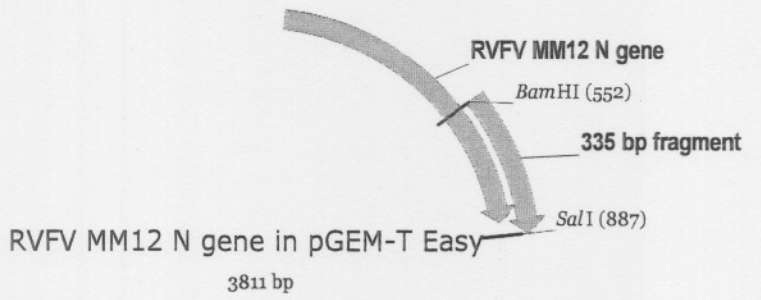
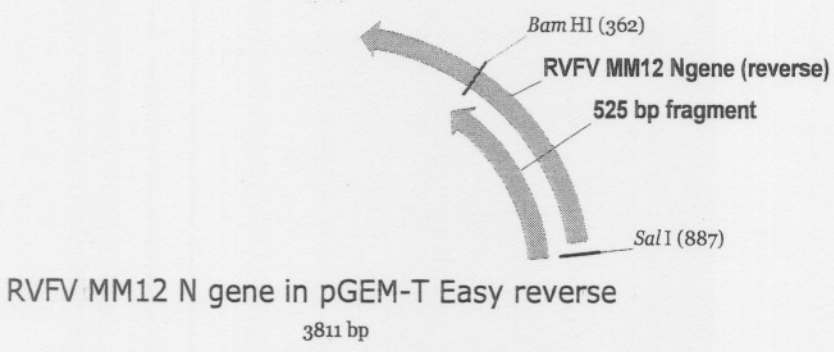


Figure 12. Gel electrophoretic analysis of purified plasmids after cloning the RVFV Zim688/78 N protein amplicon into pGEM-T Easy. A 5 µl volume of each 50 µl purified plasmid preparation was loaded as follows. Lanes: 1) White colony A1 from N prot1 plate; 2) White colony A2 from N prot1 plate; 3) Blue colony A4 from N prot1 plate; 4) White colony C1 from N prot1 plate; 5) Blue colony B5 from N prot1 plate; 6) White colony A5 from N prot1 plate; 7) O' GeneRuler DNA marker (Fermentas, Catalogue number SM1173); 8) Bulls-eye B1 from N prot1 plate; 9) Bulls-eye B2 from N prot1 plate; 10) White colony B4 from N prot1 plate; 11) White colony C3 from N prot1 plate; 12) White colony C5 from N prot1 plate; 13) Blue colony E2 from background control plate; 14) Bulls-eye E3 from background control plate; 15) Bulls-eye E4 from positive control plate; 16) White colony E5 from positive control plate; 17) White colony F1 from N prot 1 plate; 18) White colony F2 from N prot1 plate; 19) White colony F3 from N prot1; 20) O' GeneRuler DNA marker (Fermentas, Catalogue number SM1173) ; 21) White colony F4 from N prot2 plate; 22) White colony F5 from N prot2 plate; 23) Bulls-eye G1 from N prot2; 24) White colony G2 from N prot2; 25) White colony G4 from N prot1 plate and 26) Bulls-eye G5 from N prot1 plate.

To further confirm the presence and orientation of cloning, eight different purified plasmid preparations (a representative of each colony colour) were chosen for restriction analysis according to their migration patterns in Figure 12. Two restriction enzymes were chosen: SalI was chosen because pGEM-T Easy has a recognition site for this enzyme in its multiple cloning site but the RVFV MM12 N protein sequence (used as reference) does not; and BamHI was chosen because it cuts the RVFV MM12 N protein sequence only once in an asymmetric way while pGEM-T Easy does not have a BamHI site. A double digest with BamHI and SalI should identify the presence and orientation of the cloned N gene. The two possible plasmid maps that would result when the reference N gene sequence, RVFV MM12, is cloned into pGEM-T Easy is depicted in Figure 13.

Figure 13. Possible plasmid maps that would result when the reference N gene sequence, RVFV MM12, is cloned into pGEM-T Easy





The selected plasmid preparations and descriptions from the colonies from where they originated are summarized in Table 4.

Table 4. Plasmids selected for restriction enzyme analysis

Colony number	Colour on IPTG/X-gal	Plasmid purification kit	Description
B5b	Blue	PeQLab	pGEM-T Easy without insert
E5b	White	QIAgen	pGEM-T Easy with control insert
A1b	White	QIAgen	pGEM-T Easy ligated with RVFV Zim688/78 N protein amplicon
C1	White	PeQLab	pGEM-T Easy ligated with RVFV Zim688/78 N protein amplicon
F4b	White	QIAgen	pGEM-T Easy ligated with RVFV Zim688/78 N protein amplicon
A2b	White	QIAgen	pGEM-T Easy ligated with RVFV Zim688/78 N protein amplicon
B1b	Bulls-eye	QIAgen	pGEM-T Easy ligated with RVFV Zim688/78 N protein amplicon
G1b	Bulls-eye	QIAgen	pGEM-T Easy ligated with RVFV Zim688/78 N protein amplicon

Each sample was subjected to three restriction reactions as described in section MATERIALS AND METHODS (2.2.13): two single digestions with BamHI and SalI respectively, and a double digestion with BamHI and SalI (according to the instructions obtained from www.fermentas.com). A 10 µl volume of each 25 µl digestion reaction was analyzed by gel electrophoresis on a 1.0% gel depicted in Figure 14.

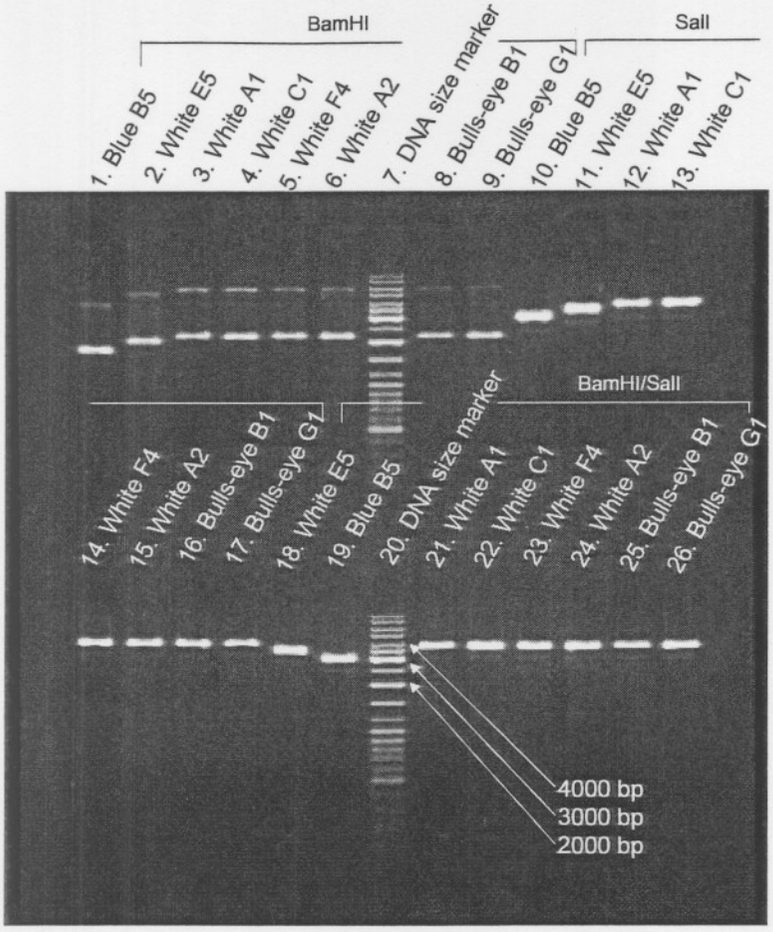


Figure 14. Gel electrophoretic analysis of restriction enzyme analysis of plasmids from the cloning experiment. A 10 µl volume of each 25 µl digestion reaction was loaded as follows. Lanes: Digested with BamHI 1) Blue colony B5 from N prot1 plate; 2) White colony E5 from positive control plate; 3) White colony Al from N prot1 plate; 4) White colony C1 from N prot1 plate; 5) White colony F4 from N prot2 plate; 6) White colony A2 from N prot1 plate; 7) O' GeneRuler DNA marker (Fermentas, Catalogue number SM1173); 8) Bulls-eye B1 from N prot1 plate; 9) Bulls-eye G1 from N prot2 plate; Digested with SalI 10) Blue colony B5 from N prot1 plate; 11) White colony E5 from positive control plate; 12) White colony Al from N prot1 plate; 13) Blue White colony C1 from N prot1 plate; 14) White colony F4 from N prot2 plate; 15) White colony A2 from N prot1 plate; 16) Bulls-eye B1 from N prot1 plate; 17) Bulls-eye G1 from N prot2 plate; Double digested with BamHI and SalI 18) White colony E5 from positive control plate; 19) Blue colony B5 from N prot1 plate; 20) O' GeneRuler DNA marker (Fermentas, Catalogue number SM1173); 21) White colony Al from N prot1 plate; 22) Blue White colony C1 from N prot1 plate; 23) White colony F4 from N prot2 plate; 24) White colony A2 from N prot1 plate; 25) Bulls-eye B1 from N prot1 plate and 26) Bulls-eye G1 from N prot2 plate.

The results obtained from the restriction analysis (Figure 14) differ from the expected results for the RVFV MM12 strain that was used to plan cloning. Since all genes encoding the N protein of the RVFV sequences published to date do not differ significantly, it was expected that the clones containing the RVFV N protein amplicon would be linearized when digested with BamHI. However, it was found that none of the Zim688/78 clones digested with BamHI was linearized (Figure 14, lanes 1 to 9). The results in lanes 1 and 2 are consistent with expected results, as lane 1 represents a plasmid from a blue colony and is, therefore, pGEM-T Easy without an insert with a BamHI restriction site. Lane 2 represents a plasmid from a white colony from the positive control plate and is, therefore, pGEM-T Easy containing the control DNA insert. It is evident from the results that the control DNA insert does not contain a restriction site for BamHI. It was expected, however, that the clones in lanes 3 to 9 would be linearized by BamHI. There are two possible explanations why BamHI did not linearize clones from the N protein amplicon. Either the BamHI restriction enzyme was not effective, or the RVFV Zim688/78 N protein gene does not have a BamHI site. This had to be resolved by analyzing the sequencing results.

Figure 14 shows that the clones in lanes 10 to 17 were linearized by SalI restriction. This is consistent with the expected results based on the assumption that, like the RVFV MM12 N gene, the Zim688/78 N gene also has no SalI site and the fact that pGEM-T Easy has a single restriction site for SalI in its multiple cloning site. It was expected that two fragments (335 bp and 3476 bp) would be visible in lanes 21 to 26 after the BamHI/SalI double digestion. However, consistent with the result above that the plasmids in Figure 14 (lanes 1 to 9), were not digested with BamHI, these clones were only linearized by SalI.

2.3.4 Sequencing and sequence analysis of gene encoding RVFV Zim688/78 N protein:

The complete sequence of the gene encoding the N protein of 7 different RVFV strains has been published (Giorgi et al., 1991; Sall et al., 1999). The sequence is of different strain isolates as listed in Table 5.

Table 5. List of available nucleic acid sequences for the gene encoding RVFV N protein of different strain isolates

Database	Database number	Strain isolate	Length	Reference
EMBL-EBI	CAA37789	MM12	738 bases	(Giorgi et al., 1991)
PubMed	AF134535	B Egy 93	735 bases	(Sall et al., 1999)
PubMed	AF134534	ZH 548	735 bases	(Sall et al., 1999)
PubMed	AF134533	H Egy 93	735 bases	(Sall et al., 1999)
PubMed	AF134532	Smithburn	735 bases	(Sall et al., 1999)
PubMed	AF134531	Clone 13	735 bases	(Sall et al., 1999)
PubMed	AF134530	MP12	735 bases	(Sall et al., 1999)

The MM12 vaccine strain sequence was used as a reference for the experiments of this study. Sequences are quite conserved between different strains of the virus but it was decided to use the MM12 strain as reference because this sequence also included the stop codon.

Sequencing of recombinant pGEM-T Easy plasmids was done at the sequencing laboratories of the Onderstepoort Veterinary Institute using M13 forward and M13 reverse primers. Gel-extraction purified N gene and diagnostic amplicons, as well as two white colony plasmids (A1 and C1) were sequenced.

To determine the termini of the amplicon of the N gene of RVFV Zim688/78, primers were designed to sequence outwards based on the consensus sequence of RVFV Zim688/78 N gene obtained from sequencing using the RVFVNPN and RVFVNPC primers designed for cloning. The sequence results were then aligned using Vector NTI to obtain the consensus sequence for the N gene amplicon and the diagnostic amplicon respectively, as well as the two clones of the N gene amplicon. The chromatograms were used in conjunction with Vector NTI to clear up uncertainties in the sequence. Chromatograms were viewed by using Chromas (Version 1.45, http://www.technelysium.com.au).

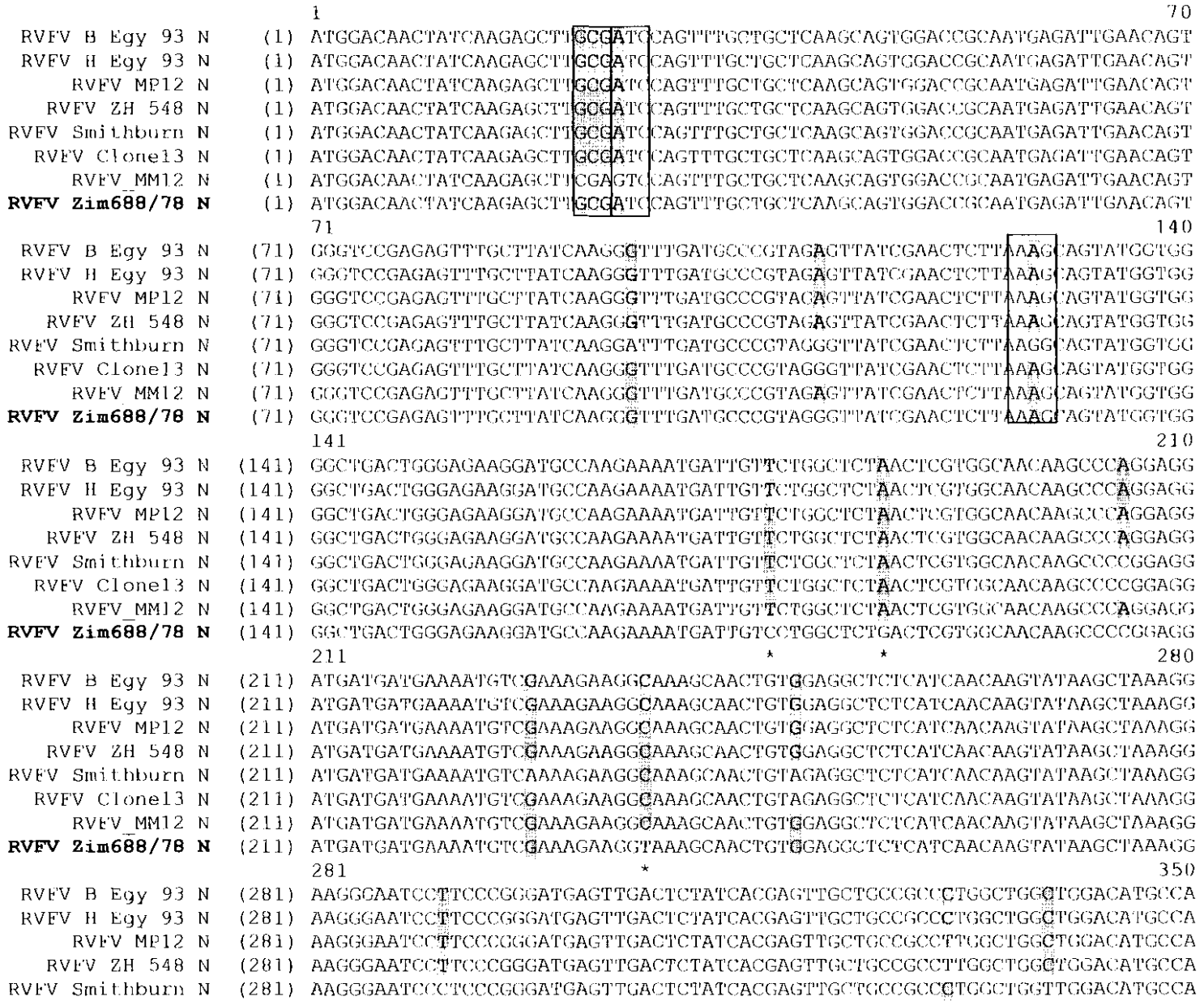
Figure 15 shows the DNA sequence of the gene encoding the N protein from the Zim688/78 RVFV strain aligned with the seven published sequences (Table 5) to identify sequence variation between strains.

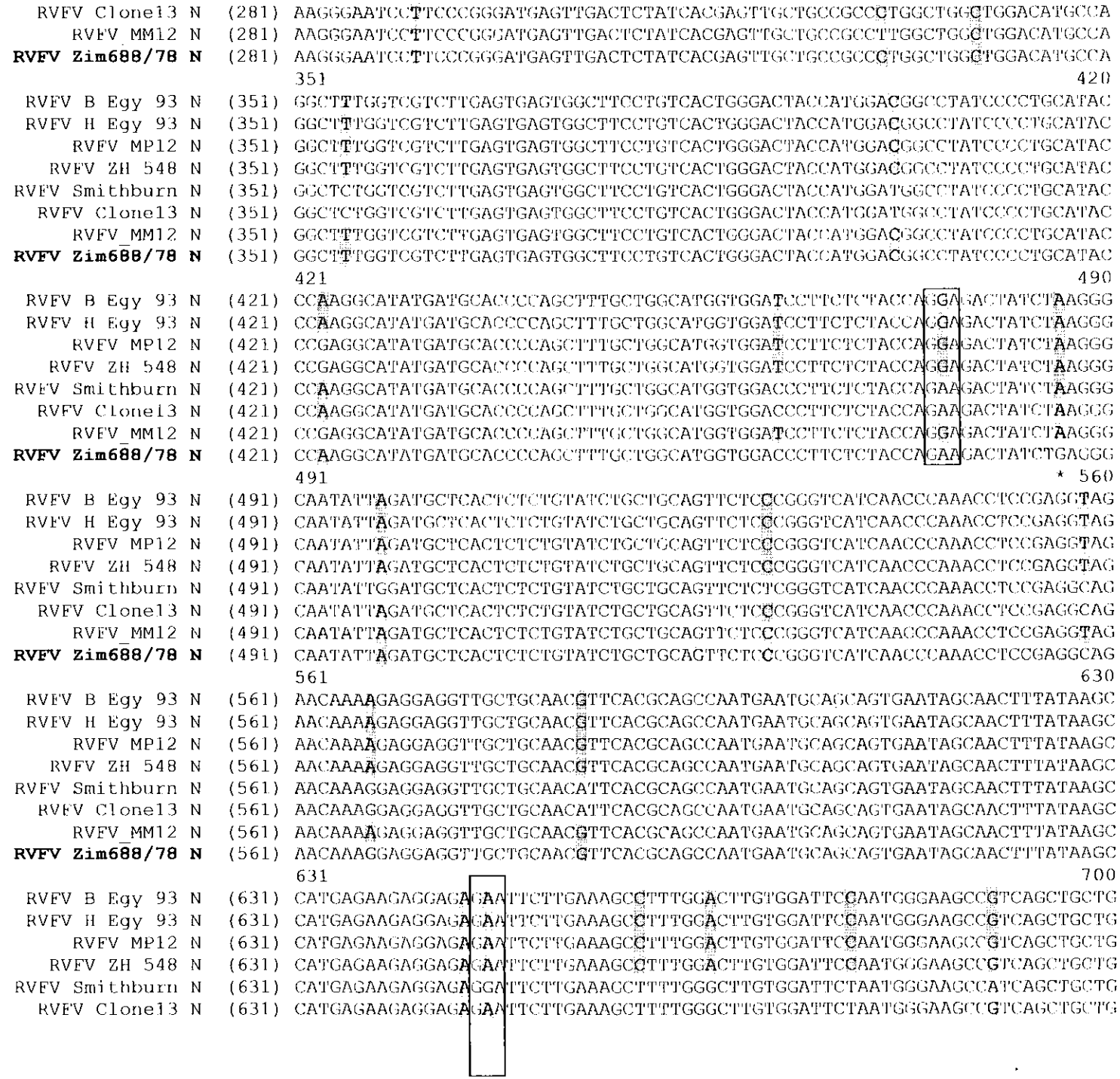
There are a total of 35 differences between the nucleic acid sequences of the eight RVFV strains, which leads to a variation of five amino acids. The stop codon is not indicated in some of the published sequences (Figure 15). The positions of the nucleic acids that lead to the variations in amino acid

sequence are indicated by the rectangular brackets in Figure 15. These results are summarized in Table 6.

Five of the 35 nucleic acid variations are unique to the RVFV Zim688/78 strain. These differences, indicated by stars under the sequence in Figure 12, are at positions 177, 186, 237, 486 and 645. None of these unique variations led to a change in the amino acid sequence.

Figure 15. The nucleic acid sequence of the gene encoding the N protein of RVFV Zim688/78, aligned with seven published sequences. Regions where all the sequences align are indicated in yellow and positions that have variations are indicated in blue. The variable base is not shaded. The variations that cause changes in the amino acid sequence are boxed. The variations unique to the ZIM688/78 strain are indicated by * under the sequence.




RVFV Clone13 N (281) AAGGGAATCCTTCCCGGGATGAGTTGACTCTATCACGAGTTGCTGCCGCCCTGGCTGGCTGGACATGCCA
RVFV MM12 N (281) AAGGGAATCCTTCCCGGGATGAGTTGACTCTATCACGAGTTGCTGCCGCCTTGGCTGGCTGGACATGCCA
RVFV Zim688/78 N (281) AAGGGAATCCTTCCCGGGATGAGTTGACTCTATCACGAGTTGCTGCCGCCCTGGCTGGCTGGACATGCCA
351 420
RVFV B Egy 93 N (351) GGCTTTGGTCGTCTTGAGTGAGTGGCTTCCTGTCACTGGGACTACCATGGACGGCCTATCCCCTGCATAC
RVFV H Egy 93 N (351) GGCTTTGGTCGTCTTGAGTGAGTGGCTTCCTGTCACTGGGACTACCATGGACGGCCTATCCCCTGCATAC
RVFV MP12 N (351) GGCTTTGGTCGTCTTGAGTGAGTGGCTTCCTGTCACTGGGACTACCATGGACGGCCTATCCCCTGCATAC
RVFV ZH 548 N (351) GGCTTTGGTCGTCTTGAGTGAGTGGCTTCCTGTCACTGGGACTACCATGGACGGCCTATCCCCTGCATAC
RVFV Smithburn N (351) GGCTCTGGTCGTCTTGAGTGAGTGGCTTCCTGTCACTGGGACTACCATGGATGGCCTATCCCCTGCATAC
RVFV Clone13 N (351) GGCTCTGGTCGTCTTGAGTGAGTGGCTTCCTGTCACTGGGACTACCATGGATGGCCTATCCCCTGCATAC
RVFV MM12 N (351) GGCTTTGGTCGTCTTGAGTGAGTGGCTTCCTGTCACTGGGACTACCATGGACGGCCTATCCCCTGCATAC
RVFV Zim688/78 N (351) GGCTTTGGTCGTCTTGAGTGAGTGGCTTCCTGTCACTGGGACTACCATGGACGGCCTATCCCCTGCATAC
421 490
RVFV B Egy 93 N (421) CCAAGGCATATGATGCACCCCAGCTTTGCTGGCATGGTGGATCCTTCTCTACCAGGAGACTATCTAAGGG
RVFV H Egy 93 N (421) CCAAGGCATATGATGCACCCCAGCTTTGCTGGCATGGTGGATCCTTCTCTACCACGAGACTATCTAAGGG
RVFV MP12 N (421) CCGAGGCATATGATGCACCCCAGCTTTGCTGGCATGGTGGATCCTTCTCTACCAGGAGACTATCTAAGGG
RVFV ZH 548 N (421) CCGAGGCATATGATGCACCCCAGCTTTGCTGGCATGGTGGATCCTTCTCTACCAGGAGACTATCTAAGGG
RVFV Smithburn N (421) CCAAGGCATATGATGCACCCCAGCTTTGCTGGCATGGTGGACCCTTCTCTACCAGAAGACTATCTAAGGG
RVFV Clone13 N (421) CCAAGGCATATGATGCACCCCAGCTTTGCTGGCATGGTGGACCCTTCTCTACCAGAAGACTATCTAAGGG
RVFV MM12 N (421) CCGAGGCATATGATGCACCCCAGCTTTGCTGGCATGGTGGATCCTTCTCTACCAGGAGACTATCTAAGGG
RVFV Zim688/78 N (421) CCAAGGCATATGATGCACCCCAGCTTTGCTGGCATGGTGGACCCTTCTCTACCAGAAGACTATCTGAGGG
491 * 560
RVFV B Egy 93 N (491) CAATATTAGATGCTCACTCTCTGTATCTGCTGCAGTTCTCCCGGGTCATCAACCCAAACCTCCGAGCTAG
RVFV H Egy 93 N (491) CAATATTAGATGCTCACTCTCTGTATCTGCTGCAGTTCTCCCGGGTCATCAACCCAAACCTCCGAGGTAG
RVFV MP12 N (491) CAATATTAGATGCTCACTCTCTGTATCTGCTGCAGTTCTCCCGGGTCATCAACCCAAACCTCCGAGGTAG
RVFV ZH 548 N (491) CAATATTAGATGCTCACTCTCTGTATCTGCTGCAGTTCTCCCGGGTCATCAACCCAAACCTCCGAGGTAG
RVFV Smithburn N (491) CAATATTGGATGCTCACTCTCTGTATCTGCTGCAGTTCTCTCGGGTCATCAACCCAAACCTCCGAGGCAG
RVFV Clone13 N (491) CAATATTAGATGCTCACTCTCTGTATCTGCTGCAGTTCTCCCGGGTCATCAACCCAAACCTCCGAGGCAG
RVFV MM12 N (491) CAATATTAGATGCTCACTCTCTGTATCTGCTGCAGTTCTCCCGGGTCATCAACCCAAACCTCCGAGGTAG
RVFV Zim688/78 N (491) CAATATTAGATGCTCACTCTCTGTATCTGCTGCAGTTCTCCCGGGTCATCAACCCAAACCTCCGAGGCAG
561 630
RVFV B Egy 93 N (561) AACAAAAGAGGAGGTTGCTGCAACGTTCACGCAGCCAATGAATGCAGCAGTGAATAGCAACTTTATAAGC
RVFV H Egy 93 N (561) AACAAAAGAGGAGGTTGCTGCAACGTTCACGCAGCCAATGAATGCAGCAGTGAATAGCAACTTTATAAGC
RVFV MP12 N (561) AACAAAAGAGGAGGTTGCTGCAACGTTCACGCAGCCAATGAATGCAGCAGTGAATAGCAACTTTATAAGC
RVFV ZH 548 N (561) AACAAAAGAGGAGGTTGCTGCAACGTTCACGCAGCCAATGAATGCAGCAGTGAATAGCAACTTTATAAGC
RVFV Smithburn N (561) AACAAAGGAGGAGGTTGCTGCAACATTCACGCAGCCAATGAATGCAGCAGTGAATAGCAACTTTATAAGC
RVFV Clone13 N (561) AACAAAGGAGGAGGTTGCTGCAACATTCACGCAGCCAATGAATGCAGCAGTGAATAGCAACTTTATAAGC
RVFV MM12 N (561) AACAAAAGAGGAGGTTGCTGCAACGTTCACGCAGCCAATGAATGCAGCAGTGAATAGCAACTTTATAAGC
RVFV Zim688/78 N (561) AACAAAGGAGGAGGTTGCTGCAACGTTCACGCAGCCAATGAATGCAGCAGTGAATAGCAACTTTATAAGC
631 700
RVFV B Egy 93 N (631) CATGAGAAGAGGAGAGAATTCTTGAAAGCCTTTGGACTTGTGGATTCCAATGGGAAGCCGTCAGCTGCTG
RVFV H Egy 93 N (631) CATGAGAAGAGGAGAGAATTCTTGAAAGCCTTTGGACTTGTGGATTCCAATGGGAAGCCGTCAGCTGCTG
RVFV MP12 N (631) CATGAGAAGAGGAGAGAATTCTTGAAAGCCTTTGGACTTGTGGATTCCAATGGGAAGCCGTCAGCTGCTG
RVFV ZH 548 N (631) CATGAGAAGAGGAGAGAATTCTTGAAAGCCTTTGGACTTGTGGATTCCAATGGGAAGCCGTCAGCTGCTG
RVFV Smithburn N (631) CATGAGAAGAGGAGAGGATTCTTGAAAGCTTTTGGGCTTGTGGATTCTAATGGGAAGCCATCAGCTGCTG
RVFV Clone13 N (631) CATGAGAAGAGGAGAGAATTCTTGAAAGCTTTTGGGCTTGTGGATTCTAATGGGAAGCCGTCAGCTGCTG

```
     RVFV_MM12 N   (631) CATGAGAAGAGGAGAGAAATTCTTGAAAGCCTTTGGACTTGTGGATTCCAATGGGAAGCCGTCAGCTGCTG
RVFV Zim688/78 N   (631) CATGAGAAGAGGAGGGAATTCTTGAAAGCTTTTGGGCTTGTGGATTCTAATGGGAAGCCGTCAGCTGCTG
                         701                 *                   738
  RVFV B Egy 93 N   (701) TCATGGCAGCCGCTCAGGCTTACAAGACAGCAGCC---
  RVFV H Egy 93 N   (701) TCATGGCAGCCGCTCAGGCTTACAAGACAGCAGCC---
     RVFV MP12 N    (701) TCATGGCAGCCGCTCAGGCTTACAAGACAGCAGCC---
   RVFV ZH 548 N    (701) TCATGGCAGCCGCTCAGGCTTACAAGACAGCAGCC---
RVFV Smithburn N   (701) TCATGGCAGCTGCTCAGGCGTACAAGACAGCAGCC---
  RVFV Clone13 N   (701) TCATGGCAGCTGCTCAGGCTTACAAGACAGCAGCC---
     RVFV_MM12 N   (701) TCATGGCAGCCGCTCAGGCTTACAAGACAGCAGCCTAA
RVFV Zim688/78 N   (701) TCATGGCAGCTGCTCAGGCCTACAAGACAGCAGCCTAA
```

Table 6. Summary of nucleic acid and amino acid variations between eight RVFV strains in the gene encoding the N protein

Nucleic acid variation	Position of nucleic acid variation	Amino acid variation	Position of amino acid variation
g/C	22	Ala/Arg	8
C/g	23		
g/A	24		
A/g	25	Ile/Val	9
g/A	96		
g/A	111		
A/g	128	Lys/Arg	43
T/C	177		
A/g	186		
A/C	205		
g/A	228		
C/T	237		
g/A	249		
T/C	291		
C/T	331		
C/T	339		
T/C	355		
C/T	402		
A/G	423		
T/C	462		
g/A	476	Glu/Gly	159
A/g	486		
A/g	498		
C/T	531		
T/C	558		
A/g	567		
g/A	585		
A/g	645		
A/g	647	Glu/Gly	216
C/T	660		
A/g	666		
C/T	678		
g/A	690		
C/T	711		
T/g/C	720		

The homology matrix in Table 7 shows that the gene encoding the N protein of the RVFV Zim688/78 strain is the closest related to the Clone13 strain, with 98.6% similarity. Clone13 is a naturally attenuated strain of RVFV with a large deletion in the NSs ORF.

Table 7. Homology matrix of the N gene DNA sequences of the seven published RVFV strains and the Zim688/78 strain.

RVFV MM12 N	100%					
RVFV BEgy93 N	99.2%	100%				
RVFV ZH548 N	99.5%	99.7%	100%			
RVFV HEgy93 N	99.2%	100%	99.7%	100%		
RVFV Smithburn N	95.9%	96.7%	96.5%	96.7%	100%	
RVFV Clone13 N	97.3%	98.1%	97.8%	98.1%	98.6%	100%
RVFV MP12 N	99.5%	99.7%	100%	99.7%	96.5%	97.8% 100%
RVFV Zim688/78 N	97.0%	97.8%	97.6%	97.8%	97.4%	**98.6%** 97.6% 100%

Figure 16. Sequence of the multiple cloning region of the plasmid pPJVV001 that resulted from the cloning of the gene encoding the N protein of the RVFV Zim688/78 strain into the pGEM-T Easy vector. The sequence of the gene encoding the RVFV Zim688/78 N protein sequence inserted in pGEM-T Easy according to A/T cloning is indicated in **shaded grey**. The primer sequences designed for RVFV Zim688/78 (RVFVNPN and RVFVNPC) are underlined. The restriction enzymes cutting in the multiple cloning site of pGEM-T Easy and RVFV MM12 N protein sequence are indicated in **bold**. The sequence of pGEM-T Easy is indicated in normal upper case text.

```
                ApaI          SphI                NotI
                ~~~~~~~       ~~~~~~              ~~~~~~~~
                                                  SacII
                                                  ~~~~~~~
                      AatII                 NcoI            EcoRI         HindIII
                      ~~~~~~~               ~~~~~~~         ~~~~~~        ~~~~~~~
   1  GGGCGAATTG GGCCCGACGT CGCATGCTCC CGGCCGCCAT GGCGGCCGCG GGAATTCGAT TAATTAAGCT TGATACAAAC ACTATTACAA TAATGGACAA CTATCAAGAG
      CTTGCGATCC
      CCCGCTTAAC CCGGGCTGCA GCGTACGAGG GCCGGCGGTA CCGCCGGCGC CCTTAAGCTA ATTAATTCGA ACTATGTTTG TGATAATGTT ATTACCTGTT GATAGTTCTC
      GAACGCTAGG

 121  AGTTTGCTGC TCAAGCAGTG GACCGCAATG AGATTGAACA GTGGGTCCGA GAGTTTGCTT ATCAAGGGTT TGATGCCCGT AGGGTTATCG AACTCTTAAA GCAGTATGGT
      GGGGCTGACT
      TCAAACGACG AGTTCGTCAC CTGGCGTTAC TCTAACTTGT CACCCAGGCT CTCAAACGAA TAGTTCCCAA ACTACGGGCA TCCCAATAGC TTGAGAATTT CGTCATACCA
      CCCCGACTGA

 241  GGGAGAAGGA TGCCAAGAAA ATGATTGTCC TGGCTCTGAC TCGTGGCAAC AAGCCCCGGA GGATGATGAT GAAAATGTCG AAAGAAGGTA AAGCAACTGT GGAGGCTCTC
      ATCAACAAGT
      CCCTCTTCCT ACGGTTCTTT TACTAACAGG ACCGAGACTG AGCACCGTTG TTCGGGGCCT CCTACTACTA CTTTTACAGC TTTCTTCCAT TTCGTTGACA CCTCCGAGAG
      TAGTTGTTCA

 361  ATAAGCTAAA GGAAGGGAAT CCTTCCCGGG ATGAGTTGAC TCTATCACGA GTTGCTGCCG CCCTGGCTGG CTGGACATGC CAGGCTTTGG TCGTCTTGAG TGAGTGGCTT
      CCTGTCACTG
      TATTCGATTT CCTTCCCTTA GGAAGGGCCC TACTCAACTG AGATAGTGCT CAACGACGGC GGGACCGACC GACCTGTACG GTCCGAAACC AGCAGAACTC ACTCACCGAA
      GGACAGTGAC
            NcoI                            NdeI
            ~~~~~~~                         ~~~~~~~
 481  GGACTACCAT GGACGGCCTA TCCCCTGCAT ACCCAAGGCA TATGATGCAC CCCAGCTTTG CTGGCATGGT GGACCCTTCT CTACCAGAAG ACTATCTGAG GGCAATATTA
      GATGCTCACT
      CCTGATGGTA CCTGCCGGAT AGGGGACGTA TGGGTTCCGT ATACTACGTG GGGTCGAAAC GACCGTACCA CCTGGGAAGA GATGGTCTTC TGATAGACTC CCGTTATAAT
      CTACGAGTGA
                 PstI
                 ~~~~~~
 601  CTCTGTATCT GCTGCAGTTC TCCCGGGTCA TCAACCCAAA CCTCCGAGGC AGAACAAAGG AGGAGGTTGC TGCAACGTTC ACGCAGCCAA TGAATGCAGC AGTGAATAGC
      AACTTTATAA
      GAGACATAGA CGACGTCAAG AGGGCCCAGT AGTTGGGTTT GGAGGCTCCG TCTTGTTTCC TCCTCCAACG ACGTTGCAAG TGCGTCGGTT ACTTACGTCG TCACTTATCG
      TTGAAATATT
                       EcoRI       HindIII
                       ~~~~~~~     ~~~~~~~
 721  GCCATGAGAA GAGGAGGGAA TTCTTGAAAG CTTTTGGGCT TGTGGATTCT AATGGGAAGC CGTCAGCTGC TGTCATGGCA GCTGCTCAGG CCTACAAGAC AGCAGCCTAA
      GTGGCTGCCC
      CGGTACTCTT CTCCTCCCTT AAGAACTTTC GAAAACCCGA ACACCTAAGA TTACCCTTCG GCAGTCGACG ACAGTACCGT CGACGAGTCC GGATGTTCTG TCGTCGGATT
      CACCGACGGG
                          SpeI          NotI          SalI                  BstXI
                          ~~~~~~~       ~~~~~~~~      ~~~~~~~               ~~~~~~~~~~~~
            XhoI                EcoRI          PstI          NdeI      SacI              NsiI
            ~~~~~~~             ~~~~~~~        ~~~~~~~       ~~~~~~    ~~~~~~            ~~~~~~~
 841  AGGGGCTCGA GAATTAATCA CTAGTGAATT CGCGGCCGCC TGCAGGTCGA CCATATGGGA GAGCTCCCAA CGCGTTGGAT GCATAGCTTG AGTATTCTAT AGTGTCACCT
      AAATAGCTTG
      TCCCCGAGCT CTTAATTAGT GATCACTTAA GCGCCGGCGG ACGTCCAGCT GGTATACCCT CTCGAGGGTT GCGCAACCTA CGTATCGAAC TCATAAGATA TCACAGTGGA
      TTTATCGAAC
```

The sequence of the plasmid pPJVV001 that resulted from the cloning of the gene encoding the N protein of RVFV Zim688/78 into pGEM-T Easy can be seen in Figure 16. Only the multiple cloning site of pGEM-T Easy with the cloned insert in the A/T site is depicted here. Restriction analysis in Vector NTI was done with the same restriction enzymes that were used to produce Figure 16, to enable a comparison between the restriction sites in the N protein sequence of the two strains.

From Figure 16 it can be seen that the RVFV MM12 N gene sequence has one restriction site for BamHI, but none for HindIII (except for the HindIII site engineered into the primer sequence). However, in Figure 16 it can be seen that the RVFV Zim688/78 N protein sequence has a restriction site for HindIII (plus the HindIII site in the primer sequence), but no site for BamHI. These results are consistent with the results obtained by the restriction analysis (as depicted in Figure 14) where it was found that BamHI did not cut any of the clones. The fact that the Zim688/78 RVFV strain has a restriction site for HindIII poses a problem for further expression experiments using the current clones. This is because the RVFVNPN primer was designed to have a HindIII restriction site for excision of the N protein sequence from the clone and easy insertion into pET-32a(+). Therefore, HindIII cannot be used as it will cause unwanted fragmentation of the N protein sequence.

The amino acid sequences of the gene encoding the N protein of Zim688/78 and all the published RVFV N genes were deduced and aligned to establish if the DNA sequence variation affected the open reading frame. The aa sequence of Zim688/78 is identical to that of the RVFV MP12 strain (Figure 17).

There are five amino acid differences between the N proteins of the 8 RVFV strains. The amino acid variations Ala8Arg and Ile9Val (Figure 17) are unique to the RVFV MM12 strain that I used as a reference. The Lys43Arg and Glu216Gly variations are unique to the RVFV Smithburn strain. There are three amino acid differences (Ala8Arg, Ile9Val and Glu159Gly) between the N protein of the RVFV Zim688/78 and MM12 strains.

Figure 17. The amino acid sequences of the N protein of the RVFV Zim688/78 and other published strains aligned. Locations where all the sequences align are indicated in yellow and positions that have variations are indicated in blue. The variable amino acid is not shaded.

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2.3.5 Partial sequence of the gene encoding the RVFV Zim688/78 G2 protein:

The diagnostic primers amplify a fragment of the M segment of the RVFV genome, more specifically a fragment of the glycoprotein G2. It was decided to sequence this fragment for the sake of obtaining more information about the Zim688/78 strain, which has not been sequenced previously. The DNA sequence of the diagnostic amplicon from the RVFV Zim688/78 strain was obtained and aligned with the 5 published sequences (BEgy93, HEgy93, MP12, Smithburn and ZH548, Table 9), using Vector NTI, as depicted in Figure 18.

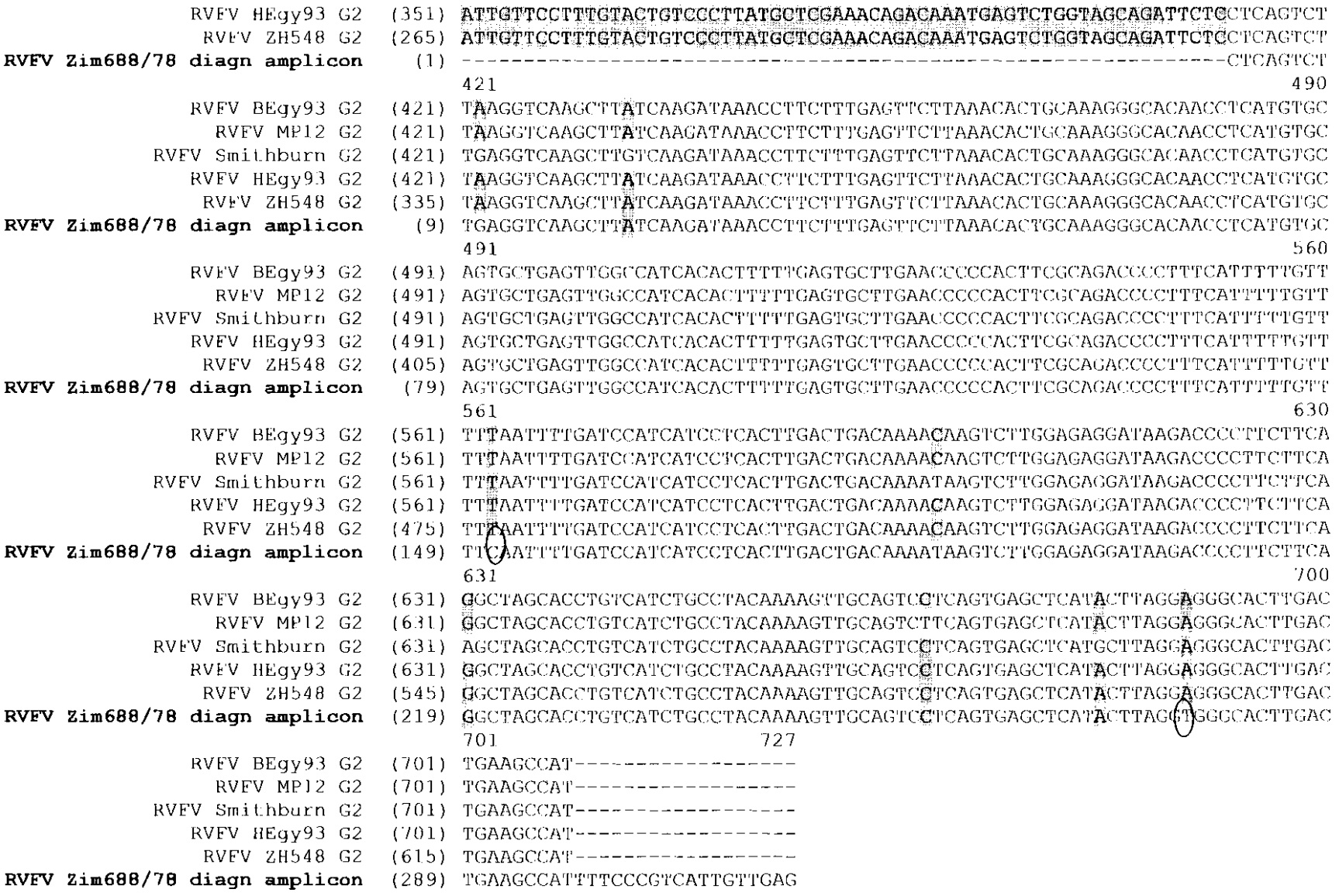
Table 8. List of available nucleic acid sequences for the gene encoding RVFV G2 protein of different strain isolates

Database	Database number	Strain isolate	Length	Reference
PubMed	AF134505	B Egy 93	727 bases	(Sall et al., 1999)
PubMed	AF134508	ZH 548	655 bases	(Sall et al., 1999)
PubMed	AF134506	H Egy 93	727 bases	(Sall et al., 1999)
PubMed	AF134501	Smithburn	738 bases	(Sall et al., 1999)
PubMed	AF134507	MP12	725 bases	(Sall et al., 1999)

As can be seen in Figure 18, the diagnostic amplicon is only a partial fragment of the gene encoding the RVFV glycoprotein G2. In the region of the known sequence of the diagnostic amplicon, there are eight variations in the nucleic acid sequences between the six strains.

Two of the eight variations in the G2 sequence are unique to the RVFV Zim688/78 strain. These are at positions 151 and 277 of the known RVFV Zim688/78 sequence, and are encircled in Figure 15. The variations at positions 434, 631 and 682 are unique to the Smithburn strain. The variation at position 668 is unique to the MP12 strain. The variations at positions 422 and 599 of the Smithburn strain are unique to the Zim688/78 and Smithburn strains (where these strains are homologous).

Figure 18. The partial nucleic acid sequence of the gene encoding the G2 glycoprotein of RVFV Zim688/78, aligned with five published sequences for the gene encoding RVFV G2 protein. Locations where all the sequences align are indicated in yellow and positions that have variations or where one of the sequences are not incomplete are indicated in blue. The variable base is not shaded. The two variations unique to the Zim688/78 strain are encircled.


RVFV HEgy93 G2 (351) ATTGTTCCTTTGTACTGTCCCTTATGCTCGAAACAGACAAATGAGTCTGGTAGCAGATTCTCCTCAGTCT
RVFV ZH548 G2 (265) ATTGTTCCTTTGTACTGTCCCTTATGCTCGAAACAGACAAATGAGTCTGGTAGCAGATTCTCCTCAGTCT
RVFV Zim688/78 diagn amplicon (1) ----------CTCAGTCT
421 490
RVFV BEgy93 G2 (421) TAAGGTCAAGCTTATCAAGATAAACCTTCTTTGAGTTCTTAAACACTGCAAAGGGCACAACCTCATGTGC
RVFV MP12 G2 (421) TAAGGTCAAGCTTATCAAGATAAACCTTCTTTGAGTTCTTAAACACTGCAAAGGGCACAACCTCATGTGC
RVFV Smithburn G2 (421) TGAGGTCAAGCTTGTCAAGATAAACCTTCTTTGAGTTCTTAAACACTGCAAAGGGCACAACCTCATGTGC
RVFV HEgy93 G2 (421) TAAGGTCAAGCTTATCAAGATAAACCTTCTTTGAGTTCTTAAACACTGCAAAGGGCACAACCTCATGTGC
RVFV ZH548 G2 (335) TAAGGTCAAGCTTATCAAGATAAACCTTCTTTGAGTTCTTAAACACTGCAAAGGGCACAACCTCATGTGC
RVFV Zim688/78 diagn amplicon (9) TGAGGTCAAGCTTATCAAGATAAACCTTCTTTGAGTTCTTAAACACTGCAAAGGGCACAACCTCATGTGC
491 560
RVFV BEgy93 G2 (491) AGTGCTGAGTTGGCCATCACACTTTTTGAGTGCTTGAACCCCCACTTCGCAGACCCCTTTCATTTTTGTT
RVFV MP12 G2 (491) AGTGCTGAGTTGGCCATCACACTTTTTGAGTGCTTGAACCCCCACTTCGCAGACCCCTTTCATTTTTGTT
RVFV Smithburn G2 (491) AGTGCTGAGTTGGCCATCACACTTTTTGAGTGCTTGAACCCCCACTTCGCAGACCCCTTTCATTTTTGTT
RVFV HEgy93 G2 (491) AGTGCTGAGTTGGCCATCACACTTTTTGAGTGCTTGAACCCCCACTTCGCAGACCCCTTTCATTTTTGTT
RVFV ZH548 G2 (405) AGTGCTGAGTTGGCCATCACACTTTTTGAGTGCTTGAACCCCCACTTCGCAGACCCCTTTCATTTTTGTT
RVFV Zim688/78 diagn amplicon (79) AGTGCTGAGTTGGCCATCACACTTTTTGAGTGCTTGAACCCCCACTTCGCAGACCCCTTTCATTTTTGTT
561 630
RVFV BEgy93 G2 (561) TTTAATTTTGATCCATCATCCTCACTTGACTGACAAAACAAGTCTTGGAGAGGATAAGACCCCTTCTTCA
RVFV MP12 G2 (561) TTTAATTTTGATCCATCATCCTCACTTGACTGACAAAACAAGTCTTGGAGAGGATAAGACCCCTTCTTCA
RVFV Smithburn G2 (561) TTTAATTTTGATCCATCATCCTCACTTGACTGACAAAATAAGTCTTGGAGAGGATAAGACCCCTTCTTCA
RVFV HEgy93 G2 (561) TTTAATTTTGATCCATCATCCTCACTTGACTGACAAAACAAGTCTTGGAGAGGATAAGACCCCTTCTTCA
RVFV ZH548 G2 (475) TTTAATTTTGATCCATCATCCTCACTTGACTGACAAAACAAGTCTTGGAGAGGATAAGACCCCTTCTTCA
RVFV Zim688/78 diagn amplicon (149) TTCAATTTTGATCCATCATCCTCACTTGACTGACAAAATAAGTCTTGGAGAGGATAAGACCCCTTCTTCA
631 700
RVFV BEgy93 G2 (631) GGCTAGCACCTGTCATCTGCCTACAAAAGTTGCAGTCCTCAGTGAGCTCATACTTAGGAGGGCACTTGAC
RVFV MP12 G2 (631) GGCTAGCACCTGTCATCTGCCTACAAAAGTTGCAGTCTTCAGTGAGCTCATACTTAGGAGGGCACTTGAC
RVFV Smithburn G2 (631) AGCTAGCACCTGTCATCTGCCTACAAAAGTTGCAGTCCTCAGTGAGCTCATGCTTAGGAGGGCACTTGAC
RVFV HEgy93 G2 (631) GGCTAGCACCTGTCATCTGCCTACAAAAGTTGCAGTCCTCAGTGAGCTCATACTTAGGAGGGCACTTGAC
RVFV ZH548 G2 (545) GGCTAGCACCTGTCATCTGCCTACAAAAGTTGCAGTCCTCAGTGAGCTCATACTTAGGAGGGCACTTGAC
RVFV Zim688/78 diagn amplicon (219) GGCTAGCACCTGTCATCTGCCTACAAAAGTTGCAGTCCTCAGTGAGCTCATACTTAGCTGGGCACTTGAC
701 727
RVFV BEgy93 G2 (701) TGAAGCCAT----------
RVFV MP12 G2 (701) TGAAGCCAT----------
RVFV Smithburn G2 (701) TGAAGCCAT----------
RVFV HEgy93 G2 (701) TGAAGCCAT----------
RVFV ZH548 G2 (615) TGAAGCCAT----------
RVFV Zim688/78 diagn amplicon (289) TGAAGCCATTTTCCCGTCATTGTTGAG

The homology matrix in Table 9 shows that with respect to the sequence of the gene encoding the glycoprotein G2, the RVFV Zim688/78 strain is the closest related to the BEgy93, HEgy93 and ZH548 strains, with 98.7% similarity.

Table 9. Homology matrix of the G2 gene sequences of five of the published strains and the Zim688/78 strain.

RVFV_BEgy93_G2	100%				
RVFV_HEgy93_G2	100%	100%			
RVFV_MP12_G2	99.9%	99.9%	100%		
RVFV_Smithburn_G2	98.1%	98.1%	97.9%	100%	
RVFV_ZH548_G2	100%	100%	99.8%	98.0%	100%
RVFV_Zim688/78_G2	**98.7%**	**98.7%**	98.3%	98.3%	**98.7%** 100%

2.4 Summary

The S segment of the RVFV genome carries the gene encoding the nucleoprotein in its negative sense. A southern African isolate of the virus from Zimbabwe (Zim688/78) was chosen for cloning, sequencing and expression. The virus was cultured successfully and subsequently the RNA was isolated. Primers were designed specifically for RT-PCR amplification of the gene encoding the N protein. The published sequence of the gene encoding the N protein of the MM12 strain (Giorgi et al., 1991) was used as a basis for primer design. The primers were designed to engineer two specific restriction enzyme sites into the opposite ends of the amplicon, for subsequent directional cloning into expression vectors. Initially a HindIII site was engineered into the 5' end of the amplicon, but after sequencing the Zim688/78 N gene amplicon, it was found that this strain of the virus has a recognition sequence for HindIII within the N gene (Figure 16) at position 656 in the ORF, which would make directional cloning of the intact gene impossible. The 5' primer was therefore designed to contain a BamHI site and the RT-PCR amplification of the N gene was successfully repeated.

The N-gene amplicon was cloned into the cloning vector pGEM-T Easy with A/T cloning. Both the amplicon as well as a clone of the pGEM-T Easy plasmid containing the N gene was sequenced. The sequencing confirmed the presence of the two restriction enzyme recognition sites at opposite ends of the amplicon, which was necessary for further cloning experiments. As positive controls during the RT-PCR reactions diagnostic primers that amplify a region of the gene encoding the glycoprotein G2 on the M segment was used. The partial G2 amplicons were also sequenced.

The sequence of the N gene from the Zim688/78 strain was compared to all 7 the published sequences of other RVFV isolates (Giorgi et al., 1991 and Sall et al., 1999). A total of 5 base variations, none of which caused amino acid changes, were unique to the Zim688/78 strain. Table 7 shows that the Zim688/78 N gene is the closest related to that of clone 13 (98.6%). Clone 13 is a naturally attenuated strain of RVFV with a large deletion in the gene encoding the non-structural protein on the S segment. The partial sequence of the gene encoding the glycoprotein G2 from the Zim688/78 strain was also compared to corresponding regions in the sequences of other published

strains. The G2 gene of the Zim688/78 strain is the closest related to the BEgy93, HEgy93 and ZH548 strains (98.7%) (Sall et al., 1999) in terms of this segment. Having cloned and determined the sequence of the gene encoding the N protein of the Zim688/78 strain it was possible to proceed with subcloning into expression plasmids as presented in the next chapter.

CHAPTER 3

BACULOVIRUS EXPRESSION OF THE RVFV ZIM688/78 NUCLEOPROTEIN

3.1 Introduction

Baculovirus expression of recombinant proteins utilizes a viral system for the expression of proteins in insect cells. Since insect cells recognize most mammalian protein-targeting sequences, mammalian proteins are generally expressed in their native forms because proteins are modified post-translationally in a very similar way as in mammalian cells. However, glycosylation of mammalian proteins in insect cells is still not achieved in the same way as in mammalian cells. The major disadvantages of baculovirus expression systems are the slow growth of insect cells, the difficulty in handling of large vectors, and laborious cell-culturing techniques (BD Biosciences, www.clontech.com). Some baculovirus systems have been developed to minimize plaque purifications by ensuring that only recombinant DNA can produce baculoviruses. Examples of such systems are the Bac-to-Bac baculovirus expression system (Invitrogen, Gibco) and the BacMagic linear DNA system (Novagen). Baculovirus systems are safe for use by humans since the virus is non-pathogenic to mammals. Baculovirus expression systems can easily be scaled up to produce large quantities of the recombinant protein. Expression levels and solubility of baculovirus expressed proteins can be affected by various combinations of expression insect host cells and the media used. Sf9 and Sf21 (*Spodoptera frugiperda*) are both good for baculovirus production and can also be used for protein expression, whereas High Five cells (*Trichoplusia ni*) usually ensures a high yield of expressed proteins.

For the purpose of this project, two commercial baculovirus expression systems are of particular interest, namely the Bac-to-Bac® Baculovirus expression system (Invitrogen) and the Bacmagic linear bacmid DNA system (Novagen).

Bac-to-Bac® Baculovirus expression system

The Bac-to-Bac® Baculovirus expression system (Luckow and Summers, 1989) is based on the transposition of an expression cassette into a specific site on a baculovirus bacmid. This differs from the traditional method of homologous recombination of infected insect cells in tissue culture. The bacmid (Figure 19) is propagated as a bacterial artificial chromosome (BAC) in *E. coli* (Wang *et. al*, 2003).

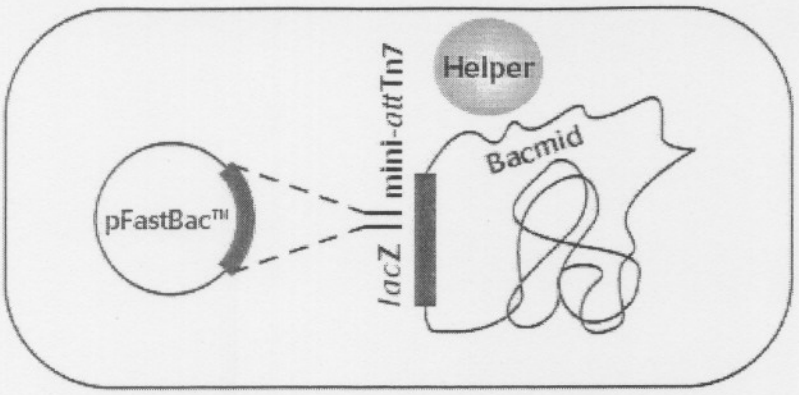


Figure 19. Representation of the Bac-to-Bac® Baculovirus expression system (Bac-to-Bac manual, Invitrogen)

The fact that the bacmid DNA is propagated in bacterial cells is a huge advantage because of the ease of handling bacteria. All bacmid manipulations are done in the bacteria up to transfection of the insect cells. Transposition is a result of the properties of the Tn7 transposon. Expression of the inserted gene is controlled by the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) polyhedron promoter (PH) which is present in the plasmid vector. The Tn7 flanks the expression cassette containing a gentamicin resistance gene and an SV40 polyadenylation signal which forms a mini Tn7. The bacmid contains a mini-*att*Tn7 target site which is used to create the recombinant bacmid. The cells containing the bacmid must also contain a helper plasmid which provides the proteins needed to facilitate transposition. When the mini-Tn7 is inserted into the mini-*att*Tn7 site on the bacmid, the expression of the LacZα peptide is disrupted which leads to white colonies when grown on plates containing X-gal and IPTG. Blue colonies represent cells that still contain the wild type bacmid.

It was found that deletion of certain genes, chitinase and v-cathepsin, in the baculovirus DNA improves the expression of secretory proteins (Kaba et al., 2004). Chitinase is a secretory protein which burdens the protein translocation machinery of the cells, thus acting as competition for recombinant secretory proteins. The protein v-cathepsin may cause proteolysis of recombinant proteins. Two expression vectors that can be used with the Bac-to-Bac system were chosen for expression of the RVFV nucleoprotein. The plasmid maps of pFastBac1 and pFastBacHT-A (one of the three reading frames) can be seen in Figures 20 and 21.

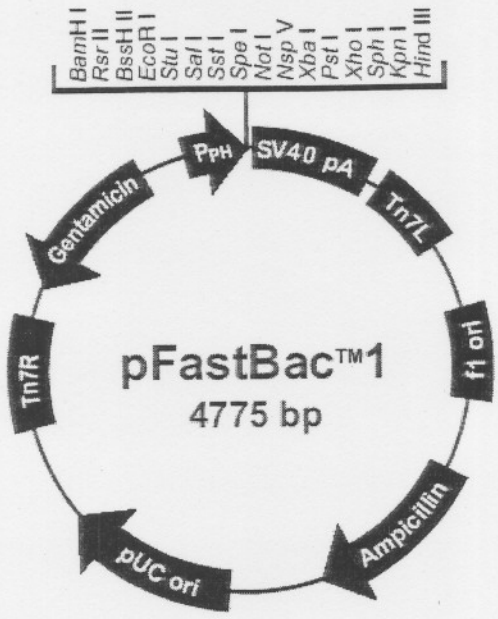


Figure 20. pFastBac1 plasmid map.

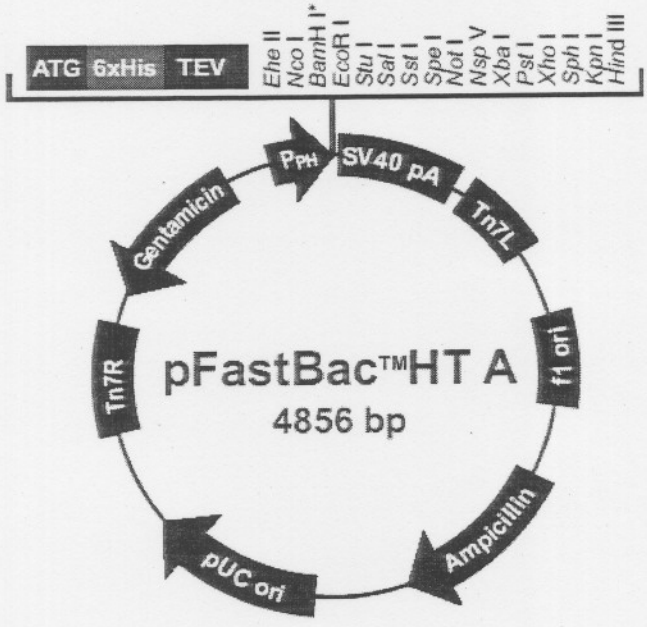


Figure 21. pFastBacHT-A plasmid map.

The two expression vectors, pFastBac1 and pFastBacHT, both have the strong polyhedrin promoter for high-level protein expression. Additionally, pFastBacHT has an N-terminal 6x Histidine tag for affinity purification of recombinant fusion proteins and a TEV protease cleavage site for removal of the tag. This vector is available in three reading frames to ensure that the target protein is in-frame with the tag. Using the expression vector that adds the affinity tag is preferential, but sometimes the fusion protein is insoluble whereas the wild-type protein without the tag may be soluble. Therefore, it was decided to use both expression vectors.

Bacmagic DNA

The advantage of using this system is that all the resultant baculoviruses are recombinant and that the cumbersome plaque purification process of recombinants is therefore eliminated. The Bacmagic DNA system (Kitts, Ayres and Possee, 1990) utilizes a linearized AcMNPV genome, with a portion of

an essential open reading frame deleted and a bacterial artificial chromosome in place of the polyhedrin coding region. This prevents non-recombinant virus from replicating in insect cells while still being able to propagate as circular DNA in bacterial cells. Homologous recombination with a compatible transfer plasmid restores the function of the open reading frame while the BAC sequence is replaced by the target sequence. We decided to use the pBACgus-1 transfer plasmid (Figure 22) as a vehicle for the RVFV N gene. This plasmid has the *gus* gene which encodes β-glucuronidase under control of the late basic protein promoter ($P_{6.9}$). The gus-gene can be utilized to verify recombinant viruses by staining with X-gluc. The vector provides a C-terminal histidine tag which can be utilized for affinity purification of the recombinant protein.

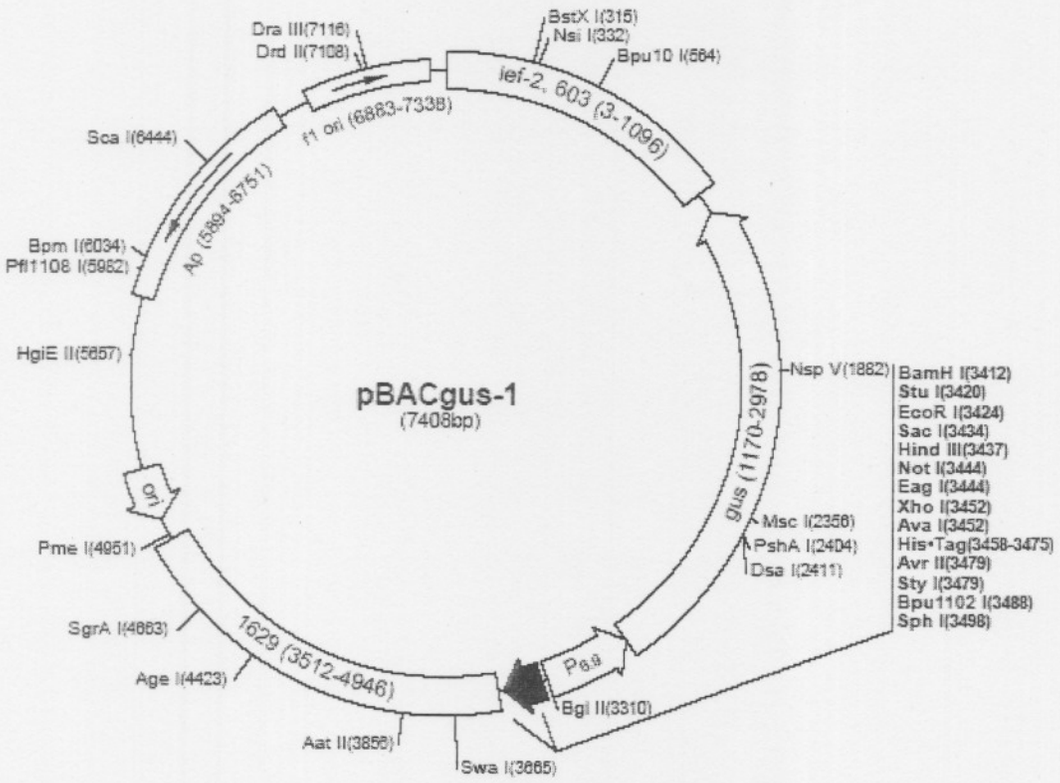


Figure 22. pBACgus-1 plasmid map

3.2 Materials and Methods

3.2.1 Cloning the gene encoding the RVFV N protein into baculovirus expression vectors:

Sequence analysis of the gene encoding the N protein of RVFV Zim688/78 indicated that BamHI and XhoI were appropriate restriction enzymes for cloning the RVFV N gene into the baculovirus expression vectors pFastBac1, pFastBac HT-B and pBACgus-1. These vectors were prepared for cloning by double digestion with BamHI and XhoI (MATERIALS AND METHODS, 2.2.13), and purified by subsequent gel-extraction (MATERIALS AND METHODS, 2.2.7). The RVFV N gene insert was prepared for cloning by double digestion with BamHI and XhoI of the RT-PCR amplicon from viral

RNA as template or recombinant plasmid (pRVFN-FBHTB) containing the RVFV N gene insert, and subsequent gel-extraction of the correct fragment.

Ligation of the RVFV N gene into baculovirus expression vectors, by means of compatible BamHI and XhoI cloning ends, was based on the methods published in the literature (Sambrook and Russell, 2001) as indicated under MATERIALS AND METHODS (2.2.10). No insert DNA was added to the ligation control reactions. For cloning into pFastBac1 (39.6 ng) and pFastBacHT-B (12 ng) different amounts of insert (0 ng, 4.42 ng, 8.84 ng and 17.68 ng) was used. For cloning into pBACgus-1 (104.8 ng) different amounts of insert (0 ng, 13.13 ng, 52.52 ng and 78.78 ng) was used.

The resultant ligation mixtures were used to transform chemically competent SURE cells (Stratagene, catalogue number 200238) using the heat-shock method (MATERIALS AND METHODS, 2.2.11). Intact pFastBac1, pFastBacHT-B or pBACgus-1 plasmid was used as transformation controls. A 100 µl volume of competent cells was transformed with 5 µl of each ligation reaction or intact plasmid. Colonies containing recombinant plasmids were selected from LB agar plates containing the appropriate antibiotics (RESULTS, 3.3.1). Recombinant plasmids were propagated, purified (MATERIALS AND METHODS, 2.2.12) and analyzed by means of agarose gel electrophoresis, restriction enzyme site analysis with appropriate restriction enzymes (RESULTS, 3.3.1) and sequencing (MATERIALS AND METHODS, 2.2.14).

3.2.2 Transposition of the RVFV N gene into Bacmid DNA:

Generation of the recombinant Bacmid DNA was done according to the instructions of the manufacturer (Bac-to-Bac® Baculovirus Expression System, Invitrogen), with slight modifications. Chemically competent Acc cells containing Bacmid was used for the transposition instead of the DH10Bac™ cells recommended by the manufacturer. Cells containing bacmid DNA was heat-shock transformed with pFastBac1 and pFastBacHT-B recombinants (pRVFN-FBAC and pRVFN-FBHTB) to facilitate transposition of the Tn7 expression cassette into the bacmid. A transformation series, consisting of 10 ng, 100 ng and 1000 ng recombinant plasmid, was used for transformation instead of the recommended 1 ng construct DNA. The plasmid pET 32(a)+ was used as transformation control instead of pUC19. pFastBac1 and pFastBacHT-B was used as positive transposition control instead of the recommended pFastBac1-Gus and pFastBacHT-CAT control plasmids. A transformation was done with no plasmid DNA to serve as a background control. X-gal (Promega, Catalogue number V3941) was used to facilitate blue-white colony selection, instead of the recommended Bluo-gal. Transformation was done in sterile 50 ml Falcon tubes instead of 15 ml round-bottom polypropylene tubes. No serial dilutions were made of the transformation mixtures. Volumes of 100 µl and 200 µl of each transformation reaction were plated on separate LB agar plates containing 50 µg/ml kanamycin, 10 µg/ml gentamicin, 10 µg/ml tetracycline, 4% IPTG and 32 µg/ml X-gal. Tetracycline was used at a higher concentration (10 µg/ml) than the recommended 7 µg/ml (A.C. Potgieter, personal communication). This helps the bacmid-containing cells to retain the helper plasmid for transposition that confers resistance to tetracycline.

Blue and white colonies were counted on each plate after 48 hours of growth. Blue and white colonies were picked up and inoculated in 3 ml LB media containing kanamycin (50 µg/ml), gentamicin (10 µg/ml) and tetracycline (10 µg/ml). The cultures were grown overnight at 37°C shaking at 200 rpm for aeration. Recombinant bacmid was purified from the overnight cultures for analysis.

3.2.3 Isolation of recombinant bacmid DNA:

The method for isolation of recombinant bacmid DNA was adapted from a method developed in the laboratory of Prof. Just Vlak (Just Vlak, personal communication, Laboratory of Virology, Wageningen University, The Netherlands).

The cells of 3 ml overnight cell cultures were collected by centrifugation at 8000 rpm for 1 minute. The supernatant was removed and the cells were resuspended in 200 µl GTE buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris/HCl pH 8, 0.1 mg/ml Rnase) in a 1.5 ml Eppendorf tube. A volume of 400 µl 0.2 M NaOH/1%SDS solution was added to each sample, mixed and incubated for 5 minutes. The tube was inverted several times after adding 300 µl chilled KAc solution (60 ml 5 M potassium acetate pH 4.8-5.2, 28.5 ml ddH_2O and 11.5 ml glacial acetic acid) and kept on ice for 5 minutes. The suspension was centrifuged at 13200 rpm in a benchtop centrifuge for 8 minutes and the supernatant transferred to a new tube. A volume of 800 µl ice-cold isopropanol was added to precipitate the DNA. The DNA precipitate was recovered by centrifugation. The DNA pellet was washed with 70% ethanol, air dried and dissolved in 40 µl TE-buffer (pH 8.5). The DNA concentration and its purity were determined with the NanoDrop spectrophotometer. Further analysis was done with PCR.

3.2.4 Analysis of recombinant bacmid DNA by PCR:

PCR for the analysis of recombinant bacmid DNA was done according to the instructions as set out in the Invitrogen Bac-to-Bac® Baculovirus expression systems manual. The PCR reaction mixtures were set up as follows: 100ng bacmid DNA, 1 × Takara Taq Buffer (Takara, Catalogue number R001AM) (100 mM Tris-HCl, pH8.3, 500 mM KCl), 1.6 mM dNTPs (supplied with Takara Taq), 1.5 mM $MgCl_2$ (supplied with Takara taq), 12.5 pmoles of each appropriate primer, Takara Taq polymerase (Takara catalogue number R001AM) and H_2O to a final volume of 50 µl. The reactions were incubated in an Eppendorf Mastercycler® ep at 93°C for 3 minutes followed by 30 cycles of 94° for 45 seconds, 55°C for 45 seconds and 72°C for 5 minutes, and a final elongation cycle at 72°C for 7 minutes. The samples were held at 4°C. The primers used in the PCR reactions are set out in the table below:

Primer	Sequence	Length	Tm
M13 Forward	5' – GTTTTCCCAGTCACGAC – 3'	17mer	51.6°C
M13 Reverse	5' – CAGGAAACAGCTATGAC – 3'	17mer	46.1°C

M13 forward and M13 reverse primers were synthesized and HPLC purified by the company Metabion.

3.2.5 Transfecting insect cells:

For initial transfections and expression we used SF9 (*Spodoptera frugiperda*) cells. 6 well plates with 35 mm wells were seeded with 1.2 x 10^6 SF9 cells in a total volume of 2 ml growth media. Growth media consisted of Grace's media (Sigma, Catalogue number G9771), 10% fetal bovine serum (FBS), antibiotics (penicillin, streptomycin and fungizone), L-arginine (85 µl per 100 ml media) and pluronic F68 (0.1%). Cells were then incubated at 27°C for 1 hour for the cells to attach. Approximately 1 µg of recombinant bacmid DNA in a volume of 10 µl (from pFastBac1 and pFastBacHT-B recombinants) was placed at the bottom of polystyrene tubes. A volume of 75 µl Fugene 6 transfection reagent (Roche, Catalogue number 11815091001) was diluted to 3 ml with Grace's medium. A volume of 190 µl of this mixture was then dripped onto the DNA in each tube and incubated at room temperature for 30 minutes. A volume of 200 µl of these DNA:lipid complexes were then used to transfect the seeded cells by dripping onto the cells, and incubated at room temperature for 30 minutes. The cells were then incubated in a humidified incubator at 27°C until signs of viral infection were visible at approximately 72 hours. The transfection supernatant was collected from each well and centrifuged at 2000 rpm for 2 minutes to pellet any cells or debris. The supernatant was collected and aliquoted for storage at 4°C and further transfections.

3.2.6 Co-transfecting insect cells:

For co-transfection with Bacmagic DNA and the pRVFN-BACg1 recombinant, SF9 cells were seeded as described above using the same growth media. A volume of 5 µl of the Bacmagic DNA (100 ng) and 3.9 µl of pRVFN-BACg1 (500 ng) were added together at the bottom of a polystyrene tube. A volume of 190 µl Fugene 6 transfection reagent plus Grace's media mixture were added as described above and incubated at room temperature for 30 minutes. This 200 µl volume co-transfection mixture was dripped onto the seeded SF9 cells as described above and incubated for 30 minutes at room temperature. The cells were then incubated in a humidified incubator at 27°C until signs of viral infection were visible after 5 days. The transfection supernatant was collected after 5 days and centrifuged at 2000 rpm for 2 minutes to collect cells and debris at the bottom of the tube. The supernatant was collected and stored at 4°C for further transfections.

3.2.7 Recombinant protein expression using virus in transfection supernatant:

Plates (6 well, 35 mm) were seeded with Sf9 cells at 1.2 x 10^{-6} cells per well and kept at 27°C. A volume of 300 µl of each transfection supernatant (as mentioned above) was diluted to 500 µl with growth media and used to infect the seeded cells. The infected cells on the plates were incubated at room temperature on a gentle rocking platform for one hour. A volume of 1.5 ml of growth media was then added to each well and the plates incubated at 27°C in a humidified incubator for 72 hours. When signs of infection were visible (72 hours) the cells were loosened from the wells by pipetting and the mixture of cells and media collected in tubes. The cells were collected by centrifugation at 500

g for 2 minutes at 4°C. The supernatant was collected and kept as the P1 viral stocks. The cell pellet was washed 2 times with 1x PBS (Calbiochem, Catalogue number 524650). The cells were lysed for 1 hour with 200 µl of the lysis buffer (0.5% NP40 in 1x PBS containing Complete mini EDTA free protease inhibitors from Roche, Catalogue number 11836170001). Soluble fraction proteins of the lysates were collected by centrifugation at 13 000 rpm for 3 minutes and collection of the supernatant. Total fraction proteins as well as soluble fraction proteins were analyzed by means of SDS-PAGE.

3.2.8 SDS-Polyacrylamide gel electrophoresis:

For analysis of proteins, SDS-PAGE analyses were done as described in Current Protocols in Molecular Biology (Gallagher, 1999). The separating gels were prepared to a final acrylamide concentration of 10% and consisted of the following: a volume of 5 ml of a 30% acrylamide/0.8% bisacrylamide stock solution, a volume of 3.75 ml 4x Tris-Cl/SDS pH 8.8 stock solution, 6.25 ml H_2O, 0.05 ml 10% (w/v) ammonium persulfate and 0.01 ml TEMED. All the reagents were added together and degassed by passing the mixture through a 0.22 µm filter with a syringe. The gel was poured into the assembled electrophoresis apparatus (BIORAD mini Protean, 76mm x 70mm) and a layer of H_2O-saturated isobutyl alcohol poured on top before polymerization at room temperature for 1 hour.

The H_2O-saturated isobutyl alcohol layer was poured of and the top of the separating gel rinsed with 1x Tris-Cl/SDS pH 8.8. The stacking gels were prepared to a final acrylamide concentration of 3.9% and consisted of the following: a volume of 0.65 ml of a 30% acrylamide/0.8% bisacrylamide stock solution, a volume of 1.25 ml 4x Tris-Cl/SDS pH 6.8 stock solution, 3.05 ml H_2O, 0.025 ml 10% (w/v) ammonium persulfate and 0.005 ml TEMED. All the reagents were added together and degassed by passing the mixture through a 0.22 µm filter with a syringe. The stacking gel was poured on top of the separating gel and the comb inserted before polymerization at room temperature for 1 hour. The tanks of the electrophoresis apparatus was filled with 1x SDS electrophoresis buffer (stock 5x buffer: 15.1 g Tris base, 72 g glycine, 5 g SDS and H_2O to a final volume of 1000 ml). Protein samples were diluted 1:1 (v/v) in a 2x SDS sample buffer (25 ml 4x Tris-Cl/SDS pH 6.8, 20 ml glycerol, 4 g SDS, 2 ml 2-mercaptoethanol, 1 mg bromophenol blue and H_2O to a final volume of 100 ml) and boiled for 3 minutes at 100°C. Samples were electrophoresed at 120 V until the bromophenol blue migrated to the bottom of the separating gel.

SDS-PAGE gels were stained according to the method of Fairbanks (Wong et al., 1999). Gels are placed in a microwavable holder with enough Fairbanks A staining solution (0.05% Coomassie, 25% isopropanol, 10% acetic acid) to cover the gel (100 ml). The gel is then heated in a microwave until the staining solution reaches boiling point (2 minutes) and cooled to room temperature for 30 minutes with gentle shaking. The staining solution was discarded and the gel rinsed with distilled water. To save time, the gels were not stained further with Fairbanks B and C staining solutions. The gels were destained with 100 ml Fairbanks D destaining solution (10% acetic acid) by heating in the microwave to boiling point (1.5 minutes), and cooling to room temperature for 30 minutes with gentle shaking. The gels were visualized by white light illuminations using a Gel-documentation system (Syngene ChemiGenius Bio-Imaging System) and GeneSnap software (Syngene, England).

3.3 Results

3.3.1 Cloning of the RVFV Zim688/78 N gene into baculovirus expression vectors pFastBac1, pFastBacHT-B and pBACgus-1:

The primers used for amplification of the RVFV Zim688/78 N gene with RT-PCR from viral RNA were designed to contain restriction sites for BamHI and XhoI creating these sites at the 3' and 5' ends of the gene's amplicon respectively. They were designed in this manner to facilitate directional insertion of the RVFV N gene into various compatible expression vectors.

Generating cloning compatible insert: A RVFV N gene amplicon containing BamHI and XhoI restriction enzyme sites was prepared from viral RNA. For cloning into expression vectors the amplicon was double digested with BamHI and XhoI. Large-scale 100 µl volume restriction enzyme digests were performed as described under MATERIALS AND METHODS (2.2.13). A total of 3.896 µg of the N gene amplicon was double digested and purified by gel-extraction as described under MATERIALS AND METHODS (2.2.7). The yield of digested DNA after gel-extraction purification was approximately 442 ng, with a concentration of 8.84 ng/µl and the $A_{260/280}$ of 2.17. This means the DNA was purified sufficiently for further cloning.

Generating cloning compatible vectors: BamHI and XhoI double digested vectors were also prepared using large-scale restriction enzyme digestions as described under MATERIALS AND METHODS (2.2.13). A total of 1.73 µg of pFastBac1 and 1.64 µg pFastBacHT-B was double digested and purified by gel-extraction respectively as described under MATERIALS AND METHODS (2.2.7). The yield of digested DNA after gel-extraction purification was approximately 990 ng of pFastBac1 (19.8 ng/µl and $A_{260/280}$ of 1.74) and 300 ng of pFastBacHT-B (6 ng/µl and $A_{260/280}$ of 1.8). The yields of the different plasmids after double digestion and gel-extraction varied, probably because of variable amounts of DNA used in the digestion reactions and variability in the gel-extraction method from batch to batch. According to the results, the DNA was purified sufficiently for further cloning.

A BamHI/XhoI fragment containing the RVFV N gene encoding the N protein was also excised from the recombinant vector pRVFN-FBHTB containing the insert after cloning into pFastBacHT-B as described above. A total of 5.56 µg of pRVFN-FBHTB plasmid was double digested and purified by gel-extraction. The yield of digested DNA after gel-extraction purification was approximately 1.313 µg, with a concentration of 26.26 ng/µl and the $A_{260/280}$ of 1.67. This is not exceptional purity but was sufficient for further cloning.

A total of 7.38 µg pBACgus-1 was double digested and purified by gel-extraction. The yield of digested DNA after gel-extraction purification was approximately 2.62 µg of pBACgus-1 (52.4 ng/µl and $A_{260/280}$ of 1.78). According to the results, the DNA was purified sufficiently for further cloning.

The RVFV N gene was cloned into the baculovirus expression vectors (pFastBac1, pFastBacHT-B and pBACgus-1) by means of ligation of their compatible BamHI and XhoI cloning ends as described under MATERIALS AND METHODS (2.2.10).

The resultant ligation mixtures were used to transform chemically competent cells as described under MATERIALS AND METHODS (2.2.11).

Volumes of 100 µl and 200 µl of the transformation reactions with recombinant pFastBac1 and pFastBacHT-B were plated on LB-agar plates containing ampicillin (100 µg/ml) and gentamycin (7 µg/ml) and incubated overnight at 37°C. Recombinant pBACgus-1 transformation reaction was plated on LB-agar plates containing ampicillin (100 µg/ml) and incubated overnight at 37°C. Several isolated colonies were picked from these plates and used to inoculate 15 ml liquid LB media containing the same antibiotics for initial recombinant screening. All liquid media cultures were incubated overnight at 37°C while shaking at 200 rpm.

A volume of 3 ml of each overnight culture was used for plasmid purification with the QIAprep Spin Miniprep Kit (QIAgen, Catalogue number 27106) as described under MATERIALS AND METHODS (2.2.12). For initial recombinant screening, extracted plasmids from all picked colonies were analyzed by agarose gel-electrophoresis (results not shown). According to the migration patterns of plasmids from certain colonies on these gels, the colonies that contained plasmids that migrated slower than control plasmids with no insert were subjected to further screening by restriction enzyme analysis with BamHI and NdeI. The following names were given subsequently to the recombinant plasmids containing the RVFV N gene: pFastBac1 → pRVFN-FBAC; pFastBacHT-B → pRVFN-FBHTB and pBACgus-1 → pRVFN-BACg1. One representative of each recombinant plasmid (pRVFN-FBAC, pRVFN-FBHTB and pRVFN-BACg1) yielding the correct size fragments after BamHI/NdeI digestions were selected and subjected to final restriction enzyme digestions to confirm the presence of the RVFV N gene in these recombinants. The RVFV N gene insert contains a restriction site for NdeI (as can be seen in Figure 16), while the expression vectors lack such a site. Therefore, linearization with this restriction enzyme could indicate the presence of the insert in the recombinant plasmids. Furthermore, double digestion of pRVFN-FBAC, pRVFN-FBHTB and pRVFN-BACg1 with BamHI and NdeI should result in one fragment of 454 bp and a larger fragment corresponding to the remaining size of the specific recombinant plasmid. A volume of 5 µl of each digestion was analyzed by means of agarose gel-electrophoresis on 1.0% gels in TAE buffer (Sambrook and Russell, 2001), as can be seen in Figure 23, Figure 24 and Figure 25.

From Figure 23 (pRVFN-FBAC) it can be seen that both pRVFN-FBAC and pFastBac1 were linearized by digestion with BamHI (lanes 4 and 5). The linearized pFastBac1 (4775 bp) has also migrated quicker through the gel than the linearized pRVFN-FBAC (5496 bp) which indicates that pRVFN-FBAC is larger than pFastbac1, which would be expected of the RVFV N gene insert (780 bp) is present in pRVFN-FBAC. Digestion of pRVFN-FBAC with NdeI yielded a partially digested linearized plasmid (5496 bp, lane 7) while pFastBac1 was not linearized by NdeI and still yielded supercoiled DNA (lane 8). The partial digestion of pRVFN-FBHTB with NdeI is evidenced by the small band above the main band in lane 7. Double digestion of pRVFN-FBAC with BamHI and NdeI yielded the expected 454 bp fragment (lane 6), further confirming the presence of the Zim688/78 RVFV N gene in the correct orientation in pRVFN-FBAC for expression, thus with the 5' terminus closest to the promoter.

Some unexpected and unidentified bands can be seen in lanes 3, 4, 6 and 7. These bands are only present in the lanes containing the recombinant plasmid (pRVFN-FBAC). These bands are unaffected by the different restriction enzyme treatments which might indicate that they can be different supercoil forms of the specific plasmid that obstruct digestion by the enzymes. It could also indicate contaminating plasmids without BamHI or NdeI sites.

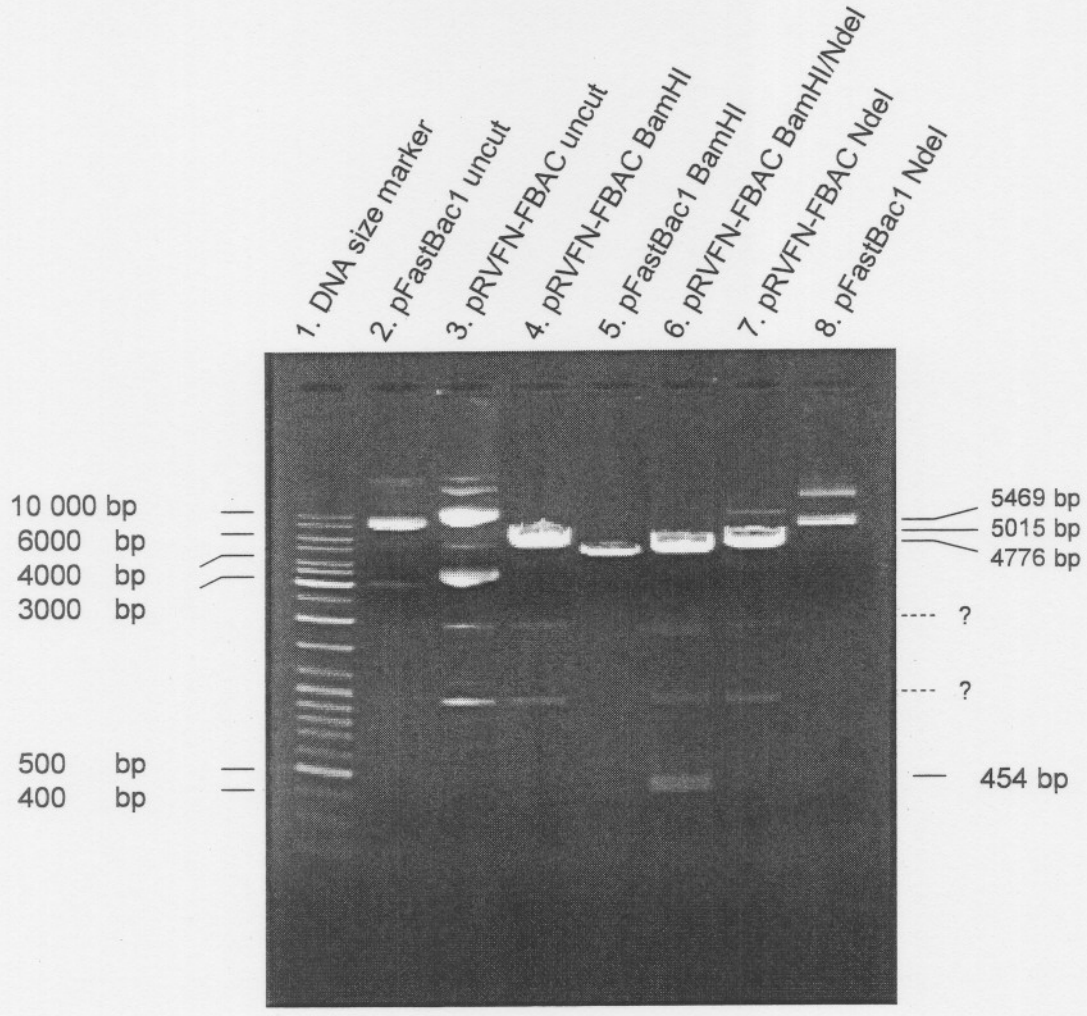


Figure 23. Agarose gel-electrophoretic analysis of restriction enzyme analysis of a plasmid obtained from the ligation of the RVFV N gene into pFastBac1 (pRVFN-FBAC). Lanes contain 5 µl of each sample: 1) O' GeneRuler DNA size marker (Fermentas, Catalogue number SM1173); 2) Intact pFastBac1 containing no insert; 3) Intact pRVFN-FBAC; 4) pRVFN-FBAC digested with BamHI; 5) pFastBac1 digested with BamHI; 6) pRVFN-FBAC digested with BamHI and NdeI; 7) pRVFN-FBAC digested with NdeI and 8) pFastBac1 digested with NdeI.

From Figure 24 (pRVFN-FBHTB) it can be seen that both pRVFN-FBHTB and pFastBacHT-B were linearized by digestion with BamHI (lanes 4 and 5). The linearized pFastBacHT-B (4857 bp) has also migrated quicker through the gel than the linearized pRVFN-FBHTB (5565 bp) which indicates that pRVFN-FBHTB is larger than pFastbacHT-B, which would be expected if the RVFV N gene insert (780 bp) is present in pRVFN-FBHTB. Digestion of pRVFN-FBHTB with NdeI also yielded linearized plasmid (5565 bp, lane 6) while pFastBacHT-B was not linearized by NdeI and still remained supercoiled DNA (lane 7, similar to lane 3). From lane 6 it would seem that NdeI did not totally linearize pRVFN-FBHTB since two bands are present, with one of the bands at the expected size of 5565 bp. Double digestion of pRVFN-FBHTB with BamHI and NdeI yielded the expected 454 bp fragment (lane 8), further confirming the presence of the Zim688/78 RVFV N gene in the correct orientation in pRVFN-FBHTB for expression, thus with the 5' terminus closest to the promoter.

The same unexpected and unidentified bands that were noticed in Figure 23 can be seen in lanes 3, 5 and 7 of Figure 24. These bands are only present in the lanes containing the pFastBacHT-B plasmid, whereas in Figure 23 the bands were only present in the recombinant pRVFN-FBAC lanes. These bands are unaffected by the different restriction enzyme treatments which might indicate that they can be different supercoil forms of the specific plasmid that obstruct digestion by the enzymes. It could also indicate contaminating plasmids without BamHI or NdeI sites.

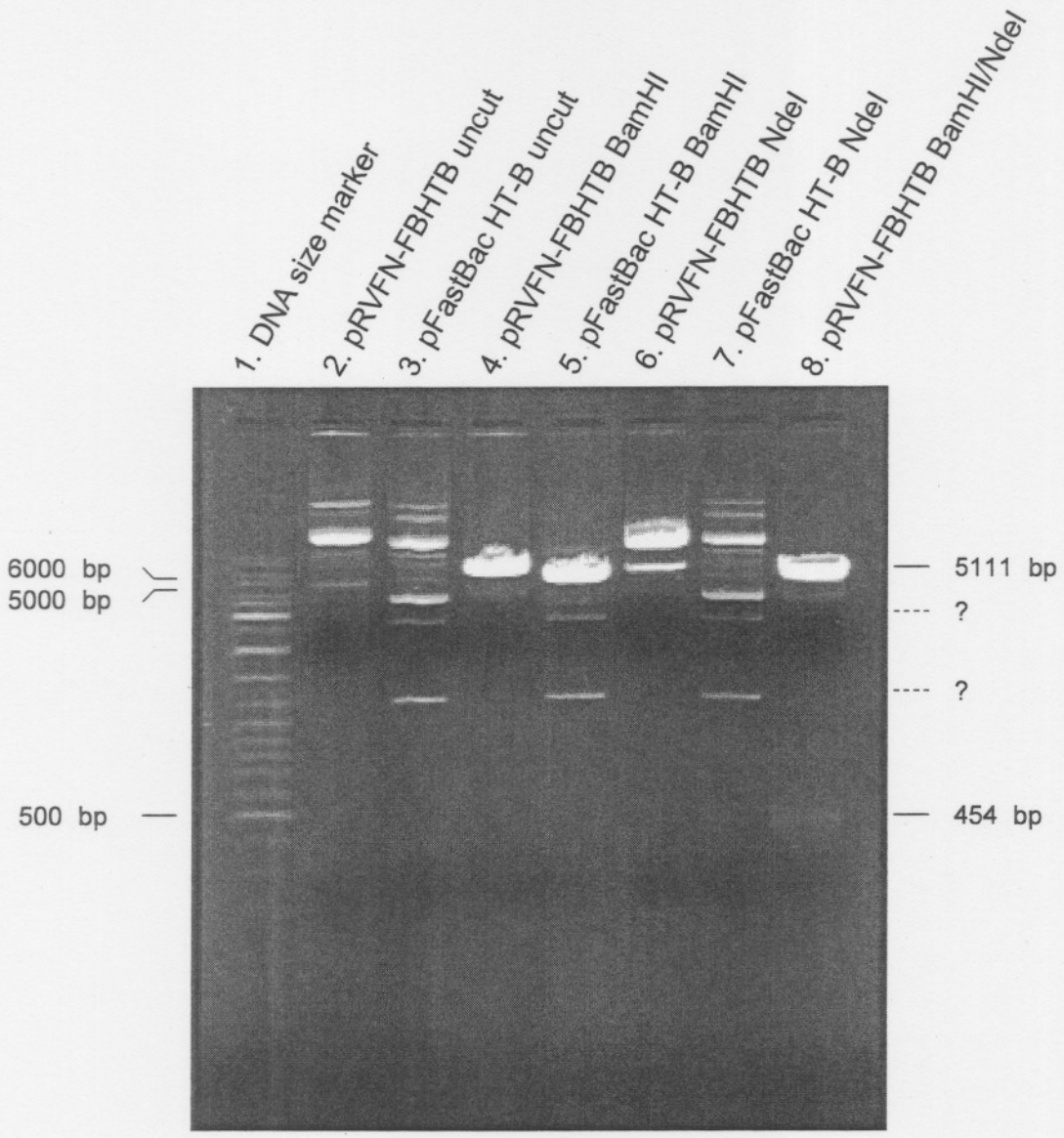


Figure 24. Agarose gel-electrophoretic analysis of restriction enzyme analysis of a plasmid obtained from the ligation of the RVFV N gene into pFastBacHT-B (pRVFN-FBHTB). Lanes contain 5 µl of each sample: 1) O' GeneRuler DNA size marker (Fermentas, Catalogue number SM1173); 2) Intact pRVFN-FBHTB; 3) Intact pFastBacHT-B; 4) pRVFN-FBHTB digested with BamHI; 5) pFastBacHT-B digested with BamHI; 6) pRVFN-FBHTB digested with NdeI; 7) pFastBacHT-B digested with NdeI and 8) pRVFN-FBHTB digested with BamHI and NdeI.

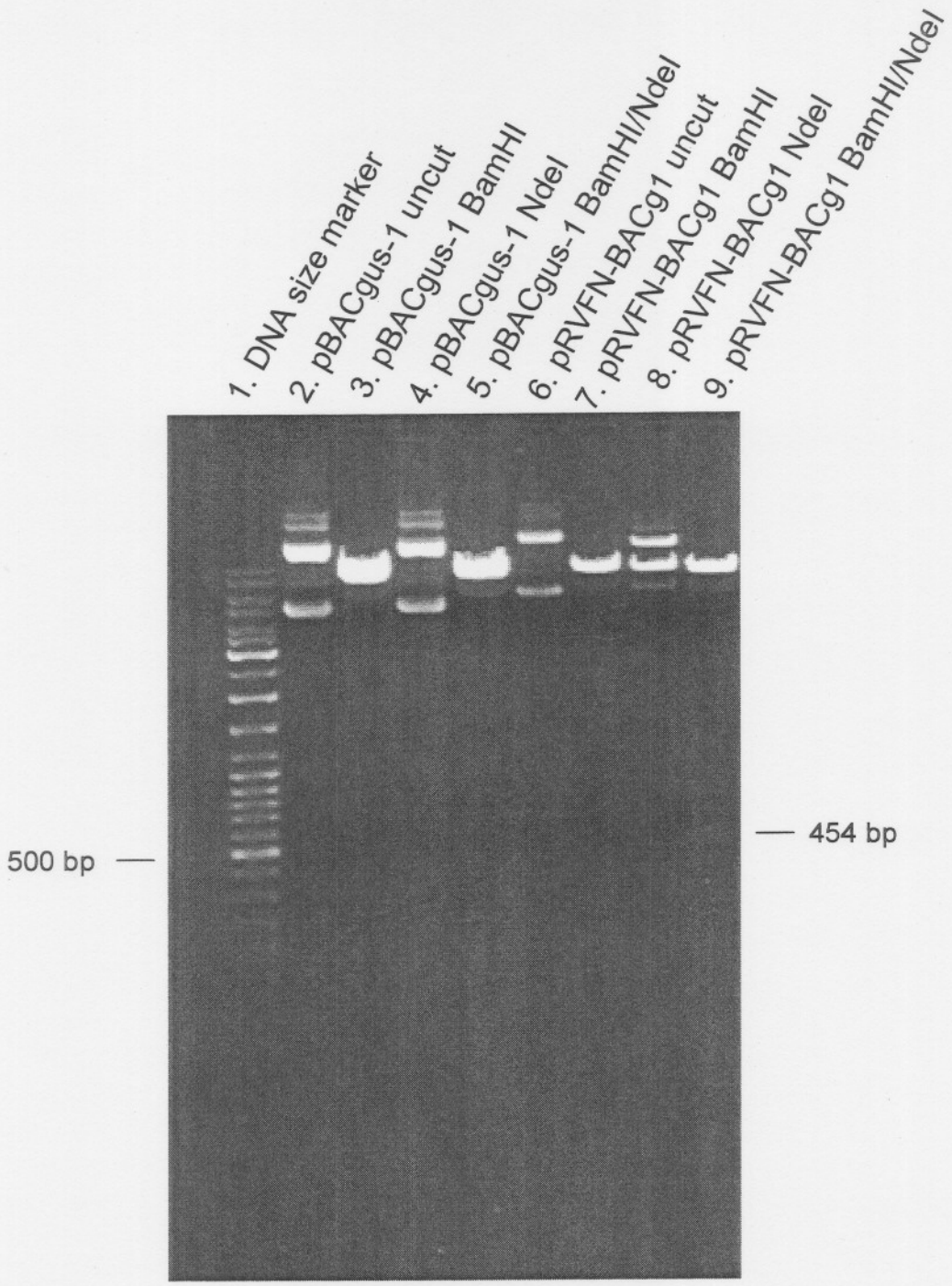


Figure 25. Agarose gel-electrophoretic analysis of restriction enzyme analysis of a plasmid obtained from the ligation of the RVFV N gene into pBACgus-1 (pRVFN-BACg1). Lanes contain 5 µl of each sample: 1) O' GeneRuler DNA size marker (Fermentas, Catalogue number SM1173); 2) Intact pBACgus-1; 3) pBACgus-1 digested with BamHI; 4) pBACgus-1 digested with NdeI; 5) pBACgus-1 digested with BamHI and NdeI; 6) Intact pRVFN-BACg1; 7) pRVFN-BACg1 digested with BamHI, 8) pRVFN-BACg1 digested with NdeI and 9) pRVFN-BACg1 digested with BamHI and NdeI.

From Figure 25 (pRVFN-BACg1) it can be seen that both pRVFN-BACg1 and pBACgus-1 were linearized by digestion with BamHI (lanes 3 and 7). The linearized pBACgus-1 (7408 bp) has also migrated quicker through the gel than the linearized pRVFN-BACg1 (8148 bp) which indicates that pRVFN-BACg1 is larger than pBACgus-1, which would be expected if the RVFV N gene insert (780 bp) is present in pRVFN-BACg1. Digestion of pRVFN-BACg1 with NdeI also yielded linearized plasmid (lane 8) while pBACgus-1 was not linearized by NdeI and still yielded supercoiled DNA (lane 4). From lane 8 it seems that the NdeI digestion was only partial (as in Figure 25) and did not totally linearize pRVFN-BACg1 since three bands are present. One of the bands aligns with the BamHI digested 8148 bp fragment. Double digestion of pRVFN-BACg1 with BamHI and NdeI yielded the expected 454 bp fragment (lane 9), further confirming the presence of the Zim688/78 RVFV N gene in the correct orientation in pRVFN-BACg1 for expression, thus with the 5' terminus closest to the promoter. Double digestion of pBACgus-1 with the same enzymes did not yield a 454 bp fragment (lane 5).

To further confirm the correct cloning and reading frame of the Zim688/78 RVFV N gene insert into the baculovirus vectors, sequence analysis was performed on the recombinant plasmid clones identified by agarose gel-electrophoresis and restriction enzyme analysis that seemed to contain the N gene. The recombinants pRVFN-FBAC and pRVFN-FBHTB were sequenced over the BamHI cloning joint with a forward primer obtained from Dr. A.C. Potgieter from OVI (5'-TggAAggCgAgCATCg -3'). The recombinant pRVFN-BACg1 was sequenced with forward and reverse primers from both sides of the insert as described in the manual of the manufacturers (Novagen). These primers, which were synthesized and HPLC purified by the company Metabion, were the T7 polyhedron promoter primer (5' – CCATCTCgCAAATAAATAAgTAT – 3') and the 1629DWN primer (5' – CTgTAAATCAACAACGcACAg – 3'). All the sequencing results confirmed that the cloning of the RVFV N gene into the various plasmids was correct (results not shown).

3.3.2 Transposition into bacmid DNA:

Transposition of the expression cassettes from pRVFN-FBAC and pRVFN-FBHTB into the bacmid DNA was done as described under MATERIALS AND METHODS (3.2.2). Blue and white colonies were counted on each plate of both recombinants. The results are depicted in Table 10 and Table 11.

No contamination took place and all colonies growing on reaction plates can be attributed to successful transformation since no colonies grew on the background control plate. Transformation was successful since the colonies on the transformation control plates grew adequately. Transposition was not as successful as expected but the presence of a minimal amount of white colonies on the reaction plates indicate that some transposition did occur. White and blue colonies were picked up for bacmid extraction and analysis.

Table 10. Blue and white colonies resulting from transposition of pRVFN-FBAC into Bacmid DNA

Plate		Number of colonies
Background control	200 µl	0
	100 µl	0
Transformation control	200 µl	> 300
	100 µl	> 300
Positive control	200 µl	> 300 blue
	100 µl	> 300 blue
1000 ng pRVFN-FBAC	200 µl	> 300 blue; 1 white
	100 µl	> 300 blue; 6 white
100 ng pRVFN-FBAC	200 µl	> 300 blue
	100 µl	> 300 blue
10 ng pRVFN-FBAC	200 µl	> 300 blue; 5 white
	100 µl	> 300 blue; 4 white

Table 11. Blue and white colonies resulting from transposition of pRVFN-FBHTB into Bacmid DNA

Plate		Number of colonies
Background control	200 µl	0
	100 µl	0
Transformation control	200 µl	128
	100 µl	109
Positive control	200 µl	73 blue
	100 µl	62 blue; 1 white
1000 ng pRVFN-FBHTB	200 µl	66 blue
	100 µl	64 blue
100 ng pRVFN-FBHTB	200 µl	55 blue; 1 white
	100 µl	73 blue; 1 bulls-eye
10 ng pRVFN-FBHTB	200 µl	87 blue; 1 white; 1 bulls-eye
	100 µl	55 blue; 1 bulls-eye
1 ng pRVFN-FBHTB	200 µl	43 blue; 1 white; 1 bulls-eye
	100 µl	20 blue

A total of 5 white colonies and one blue colony were picked from the overnight plates on which transposition reactions with pRVFN-FBAC and pRVFN-FBHTB were plated. One white and one blue colony were also picked from the transposition control plates with pFastBac1 and pFastBacHT-B to serve as controls for the subsequent PCR analysis. These colonies were used to inoculate LB-broth with the appropriate antibiotics for overnight cultures as described under MATERIALS AND METHODS (3.2.3).

The transpositions were repeated with pRVFN-FBAC and pRVFN-FBHTB at the laboratories of Onderstepoort Veterinary Institute and resultant bacmid DNA analyzed by PCR as described in MATERIALS AND METHODS (3.2.4) and below.

3.3.3 PCR analysis of recombinant Bacmid DNA:

Recombinant bacmid DNA, isolated from overnight cultures, was analysed by PCR with M13 forward and reverse primers, as described in MATERIALS AND METHODS (3.2.4), to confirm correct transposition of the expression cassette from recombinant pRVFN-FBAC and pRVFN-FBHTB vectors into the bacmid DNA. The M13 forward and reverse primer sequences flank the mini-attTn7 site where transposition takes place. Successful transposition from pFastBac1 recombinants should yield a 3080 bp fragment (2300 bp plus the size of the insert, 780 bp), while successful transposition from pFastBacHT-B recombinants should yield a 3210 bp fragment (2430 bp plus the size of the insert, 780 bp). Unsuccessful transposition (bacmid from a blue colony) should yield a 300 bp fragment. The results of the PCR analysis of recombinant bacmids from pRVFN-FBAC and pRVFN-FBHTB can be seen in Figures 26 and 27. The result of the PCR analysis of recombinant bacmid DNA from transpositions at OVI with pRVFN-FBAC and pRVFN-FBHTB can be seen in Figure 28.

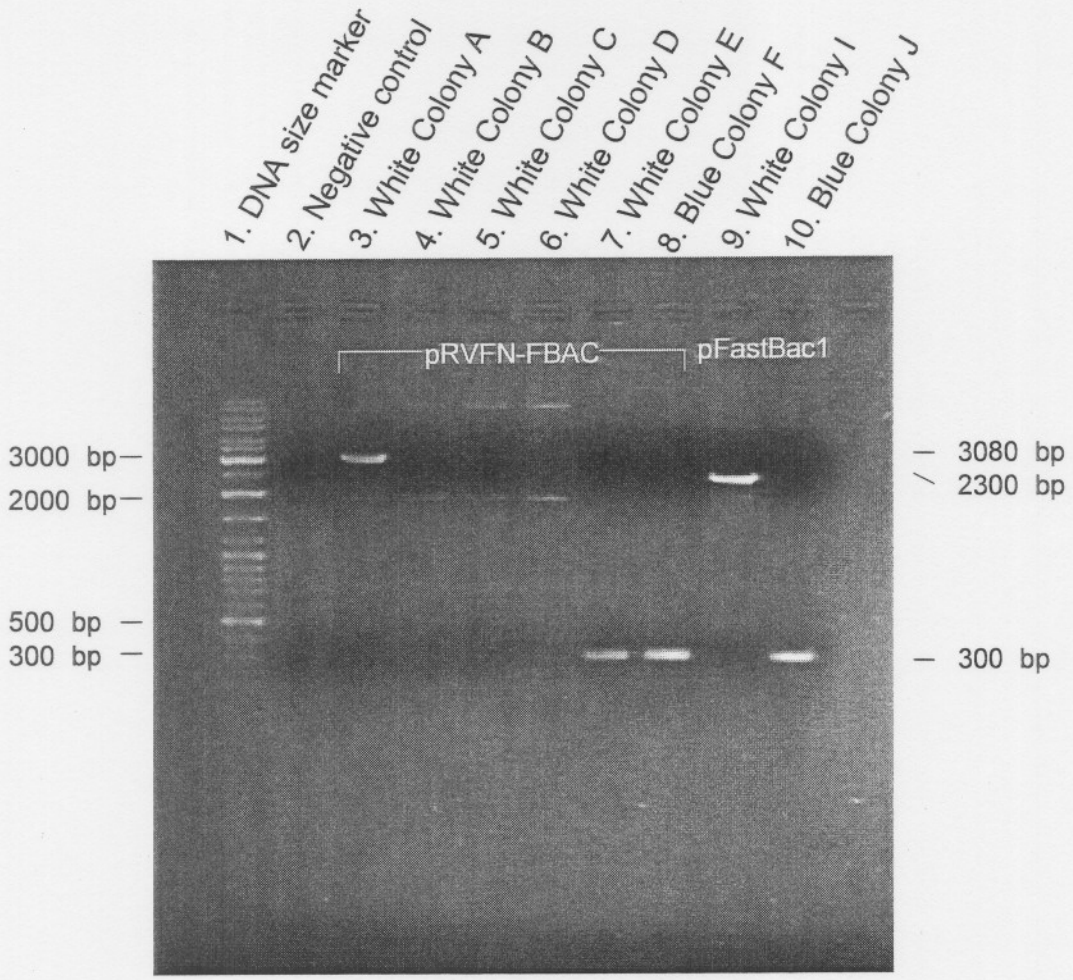


Figure 26. Agarose gel electrophoresis of recombinant bacmid PCR analysis from pRVFN-FBAC.

Lanes contain 3 µl of each 50 µl amplicon: 1) O' GeneRuler DNA size marker (Fermentas, Catalogue number SM1173); 2) Negative control, no DNA; 3) white colony A from pRVFN-FBAC transposition; 4) white colony B from pRVFN-FBAC transposition; 5) white colony C from pRVFN-FBAC transposition; 6) white colony D from pRVFN-FBAC transposition; 7) white colony E from pRVFN-FBAC transposition; 8) blue colony F from pRVFN-FBAC transposition; 9) white colony I from pFastBac1 transposition; 10) white colony J from pFastBac1 transposition.

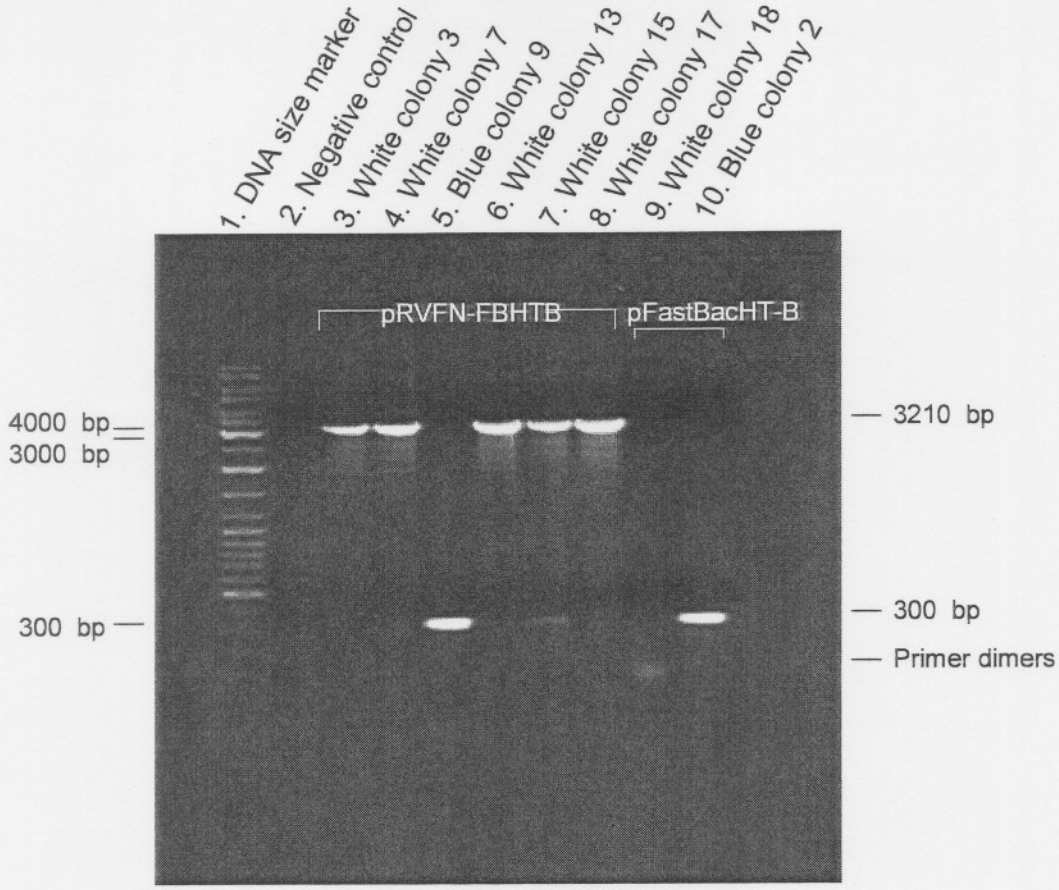


Figure 27. Agarose gel electrophoresis of recombinant bacmid PCR analysis from pRVFN-FBHTB.

Lanes contain 5 µl of each 50 µl amplicon: 1) O' GeneRuler DNA size marker (Fermentas, Catalogue number SM1173); 2) Negative control, no DNA; 3) white colony 3 from pRVFN-FBHTB transposition; 4) white colony 7 from pRVFN-FBHTB transposition; 5) blue colony 9 from pRVFN-FBHTB transposition; 6) white colony 13 from pRVFN-FBHTB transposition; 7) white colony 15 from pRVFN-FBHTB transposition; 8) white colony 17 from pRVFN-FBHTB transposition; 9) white colony 18 from pFastBacHT-B transposition; 10) blue colony 2 from pFastBacHT-B transposition.

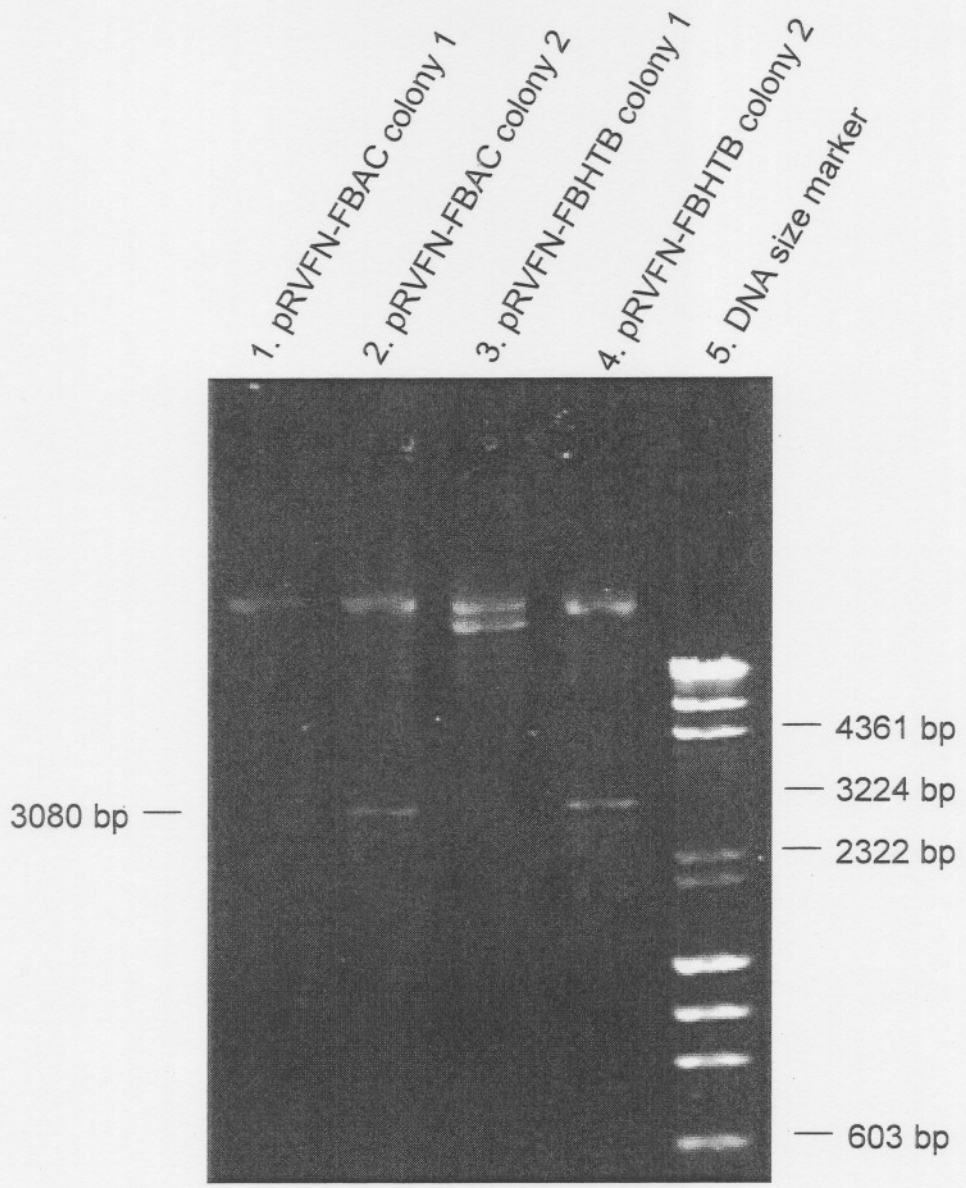


Figure 28. Agarose gel electrophoresis of recombinant bacmid PCR analysis from pRVFN-FBAC and pRVFN-FBHTB clones.

Lanes contain 2 µl of each 50 µl amplicon: 1) white colony from pRVFN-FBAC clone 1; 2) white colony from pRVFN-FBAC clone 2; 3) white colony from pRVFN-FBHTB clone 1; 4) white colony from pRVFN-FBHTB clone 2; 5) Finnzymes DNA size marker (λ DNA HindIII digest and øX174 DNA HaeIII digest, Finnzymes, Catalogue number F303SD).

According to the sizes of the bands in Figures 26 and 27, it was decided to use colony A (Figure 26) for further baculovirus expression work with pFastBac1 recombinants, and colony 13 (Figure 27) for pFastBacHT-B recombinants.

From Figure 28 it can be seen that lane 2 has a band in the range of 3080 bp which indicates that transposition was successful with the recombinant bacmid DNA of the pRVFN-FBAC from colony 2. Lane 4 has a band in the range of 3210 bp which indicates that transposition was successful with the recombinant bacmid DNA of the pRVFN-FBHTB from colony 2. Lanes 1-4 contain very high molecular weight DNA which can be seen at the top of each lane. This could indicate an overload of bacmid DNA in the PCR reactions. Lanes 1 and 3 contains only these high molecular weight bands but no bands in the region of 3000 bp, which indicates that transposition was most likely unsuccessful with these recombinant bacmids The smaller 300 bp band indicative of no transposition is not visible because the electrophoresis was too long and it migrated off the bottom of the gel. Recombinant bacmid DNA from all 4 colonies were therefore used for generating baculovirus recombinants and evaluate protein expression.

3.3.4 Baculovirus protein expression:

For proof of principle of baculovirus expression of recombinant RVFV N protein as a diagnostic antigen, recombinant baculoviruses were generated from the four clones of bacmid DNA from pRVFN-FBAC and pRVFN-FBHTB that were generated at OVI. To determine whether these recombinants expressed the RVFV N protein, the cell pellets from infected cells at 72 hours were washed with 1x PBS, and the cells lysed with the lysis buffer (0.5% NP40 in 1x PBS containing Protease inhibitors) as described under MATERIALS AND METHODS (3.2.7). The total fraction of proteins expressed in mock-infected and recombinant infected SF9 cells was lysed and analyzed by means of SDS-PAGE analysis. These results can be seen in Figure 29. Lane 1 contains the total fraction proteins from a mock infected well of SF9 cells (no transfection done). Lane 2 contains the total fraction proteins from the RVFN-FBAC colony 1 derived recombinant virus. The deduced amino acid sequence indicates that the recombinant RVFV N protein should have a molecular weight of ~28 kDa. There is a band at the correct position (28 kDa) on the gel in lane 2. Compared to the same band in lane 3, this product was much less. Thus a low level expression of RVFV N did occur with this recombinant virus. Lane 3 contains the total fraction proteins from the RVFN-FBAC colony 2 derived recombinant virus. There is a band of much higher expression at the 28 kDa size, which indicates that expression of the RVFV N protein did occur at relatively high levels with this recombinant virus. Lane 4 contains the total fraction proteins from the RVFN-FBHTB colony 2 recombinant. The deduced amino acid sequence indicates that the RVFV N protein plus the His fusion tag should have a molecular weight of ~32 kDa. There is a band at the correct position (32 kDa) on the gel in lane 4, meaning that expression did occur at relatively high levels. Lane 5 contains the total fraction proteins baculovirus recombinant derived from a blue colony Bacmid control. The fusion tag was not visible on the gel, probably due to its small size (4 kDa), which means it migrates at the front where there is not adequate resolution.

Both RVFN-FBAC baculovirus recombinants express a protein of ~28 kDa (lanes 2 and 3) which corresponds with the calculated molecular weight of 28 kDa of the RVFV N protein. However, judged from the intensities of the stained bands the recombinant 2 (lane 3) expresses this protein at a considerably higher level than recombinant 1 (lane 2). The RVFN-FBHTB baculovirus recombinant express a protein of ~32 kDa (lane 4) which corresponds with the calculated molecular weight of the RVFV N protein plus the His fusion tag. Judged from the intensity of the band in lane 4, this recombinant expressed the target protein at a lower level than recombinant 2 RVFN-FBAC.

For biological activity, it is usually required that recombinant proteins are expressed in a soluble form. To determine the solubility of the expressed RVFV N proteins, the soluble fraction proteins from all the samples (except the mock infected cells) were analyzed by means of SDS-PAGE. The results can be seen in Figure 30. A portion of the total RVFV N protein fraction from both the RVFN-FBAC recombinants was soluble (lane 1 and 2). It is not possible to determine whether the RVFV N protein expressed by the RVFN-FBTHB recombinant was soluble because the band where the protein should be is the same size as a cellular protein (see lane 4). The only way to determine this would be by a western blot.

Bacmid DNA from colonies A and 13 was sent to the commercial laboratories of the Biovac Institute (Pinelands, South-Africa) for transfections, plaque purification, optimization of protein expression and up-scaling of antigen production.

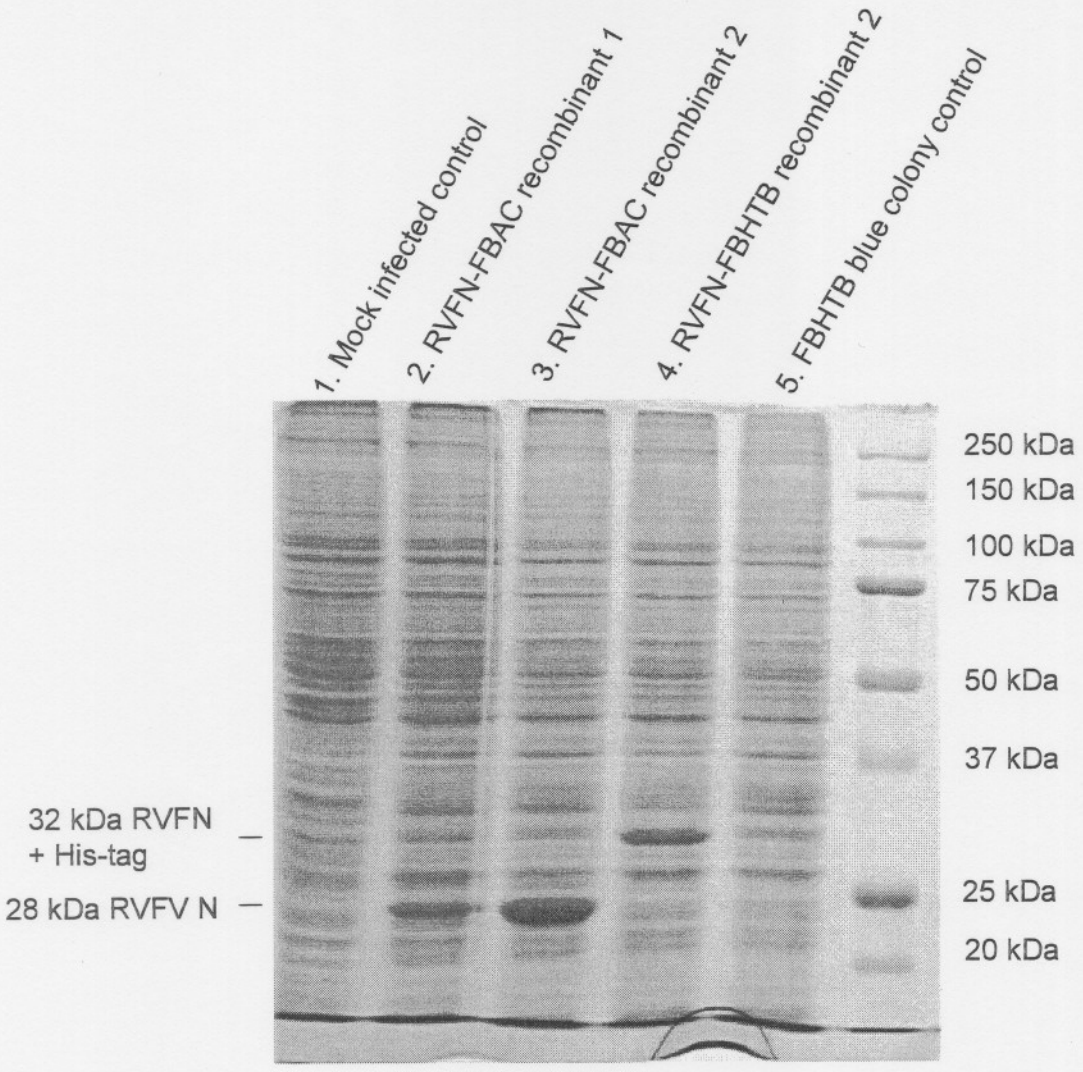


Figure 29. SDS-PAGE analysis of the total fraction of proteins expressed in SF9 cells infected with RVFN-FBAC and RVFN-FBHTB recombinant P1 baculoviruses respectively.

Lanes contain 6 µl of each sample infected with the recombinant virus as indicated: 1) Mock infected cells; 2) RVFN-FBAC recombinant 1 proteins; 3) RVFN-FBAC recombinant 2 proteins; 4) RVFN-FBHTB recombinant 2 proteins; 5) FBHTB recombinant derived from a blue colony bacmid control; 6) Protein molecular weight marker (BioRad,Catalogue number 161-0374).

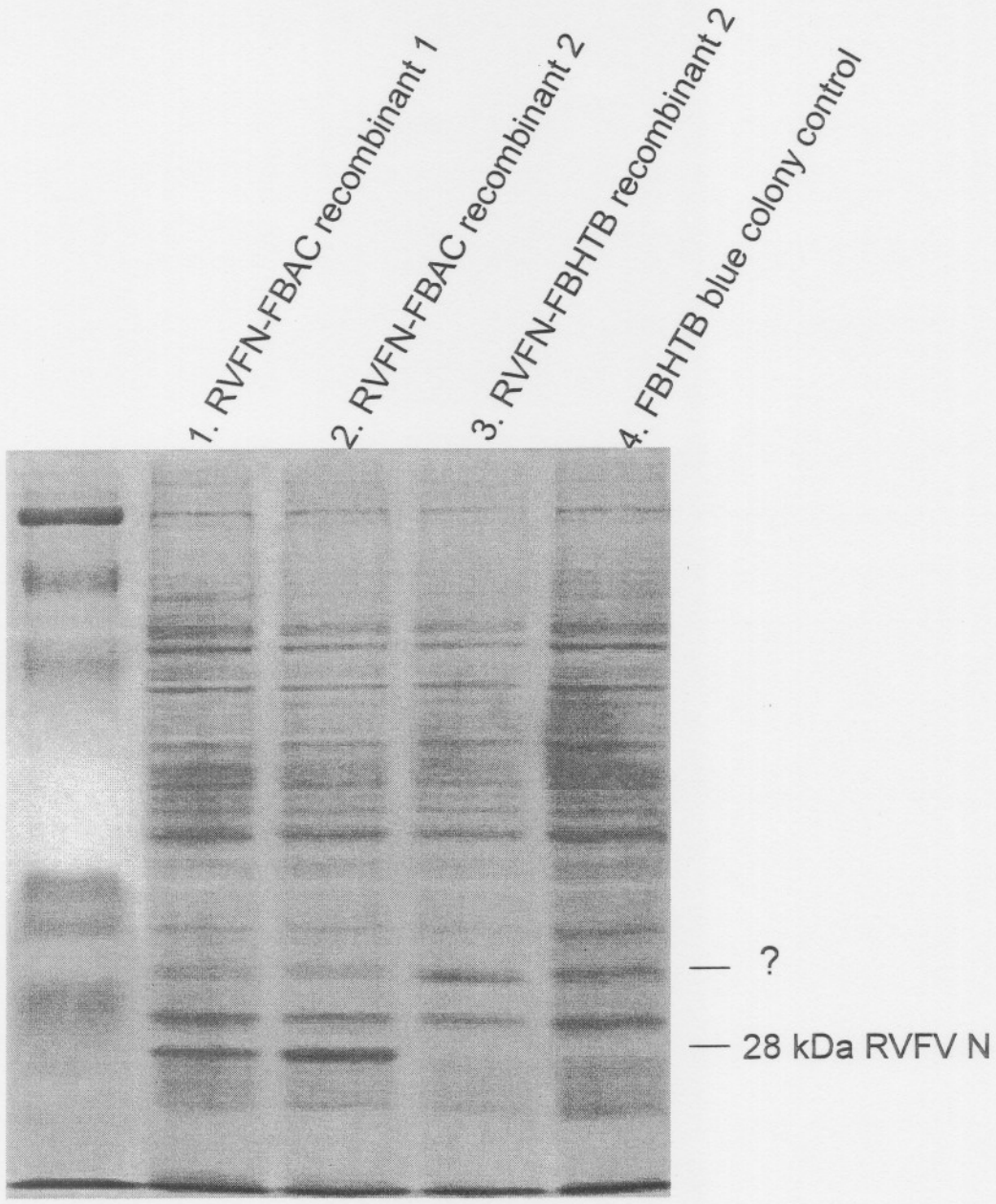


Figure 30. SDS-PAGE analysis of the soluble proteins expressed in SF9 cells infected with RVFN-FBAC and RVFN-FBHTB recombinant P1 baculoviruses respectively.

Lanes contain 6 µl of each sample infected with the recombinant virus as indicated: Protein molecular weight marker (BioRad,Catalogue number 161-0374); 1) RVFN-FBAC recombinant 1 proteins; 2) RVFN-FBAC recombinant 2 proteins; 3) RVFN-FBHTB recombinant 2 proteins; 4) FBHTB recombinant derived from a blue colony bacmid control.

3.4 Summary

The baculovirus expression system is very useful for the expression of recombinant eukaryotic antigens because its post-translational modification, especially glycosylation, is closer to that of mammalian cells than the bacterial systems (Luckow and Summers, 1989). This is usually important to obtain soluble, biologically active recombinant proteins. Scale-up is possible once the correct recombinant viruses have been produced and plaque-purified. Baculovirus expression has been used successfully in the past for the expression of recombinant RVFV antigens. Schmaljohn *et. al.* (1989) used baculovirus expression for the production of recombinant proteins (G1 and G2) from the M genome segment of RVFV. These recombinants were used to study their application as immunogens for vaccination and antigens for serological tests.

The RVFV N-gene from RVFV strain Zim688/78 was cloned into three different baculovirus expression vectors. Recombinant bacmid DNA was used to transfect SF9 insect cells to investigate expression levels of the RVFV N-protein. The level of expression from pFastBacHT-B was relatively low and it was largely insoluble (Figure 30). The level of expression from pFastBac1 was higher than from pFastBacHT-B and it was also slightly more soluble (Figure 30). Recombinant bacmid DNA from both expression vectors was sent to the commercial company, Biovac (Pinelands, South Africa), for further optimization and up-scaling of expression. Results are not yet available.

Because baculovirus expression is relatively expensive, technically demanding and time-consuming various bacterial expression systems were also investigated as described in the next chapter.

CHAPTER 4

BACTERIAL EXPRESSION OF RECOMBINANT RVFV NUCLEOPROTEIN

4.1 Introduction

The major advantages of using bacterial expression systems to produce recombinant proteins are the low costs, the fast growth-rate of bacteria, the ease of scaling-up and because expression can be regulated. One of the problems of bacterial expression systems is the lack of mammalian-like post-transcriptional modifications. There are, however, some expression vectors and certain cell-lines that contain specific genes which could be used to produce biologically active proteins. Two vector systems were chosen for bacterial expression of the RVFV nucleoprotein. The first expression vector, pGEX4T-1, makes use of the Glutathione S-transferase (GST) gene fusion system. The system utilizes inducible, high-level expression of genes as fusions with the GST protein of *Schistosoma japonicum* (Amersham catalogue), which is added as an amino terminus tag to the target protein. The GST protein has a molecular weight of 26 000 kDa and as a tag it still has enzymatic activity. Fusion proteins containing this GST protein can be purified using affinity chromatography with immobilized glutathione. The tag can be removed by site specific cleavage with the protease thrombin. Expression of inserted genes is under control of the *tac* promoter which is inducible with IPTG. The vector also contains the *lacI*^{q} gene which encodes a repressor protein that prevents expression until the addition of the inducer IPTG. The *E. coli* strain that is used with this expression system, BL21 [genotype F-, ompT, hsdS (rB-, mB-), gal], is deficient in OmpT and Lon protease production. The plasmid map of pGEX4T-1 can be seen in Figure 31.

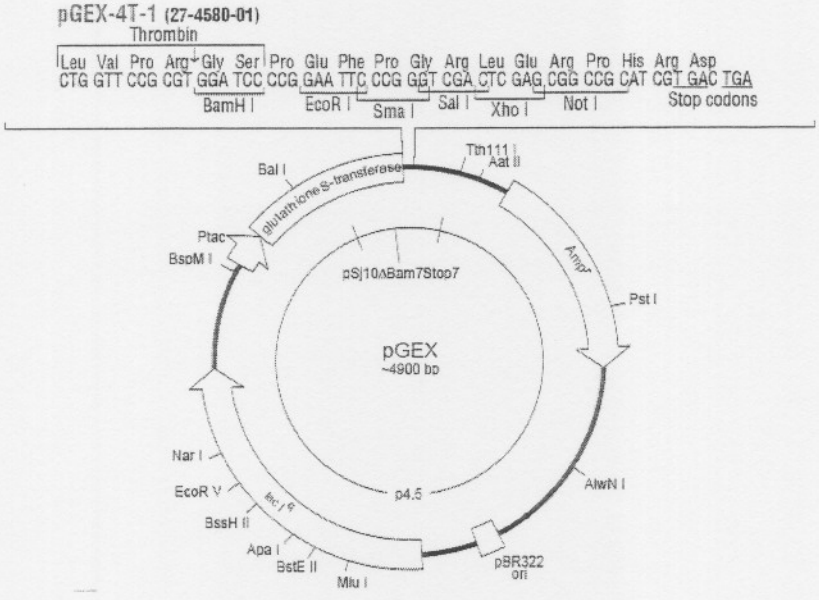


Figure 31. Plasmid map of pGEX4T-1.

The second bacterial expression vector, pET32a(+), makes use of the T7 expression system. The expression of target genes is controlled by the T7 promoter. This promoter is not recognized by *E. coli* RNA polymerase, thus no expression occurs until a source of T7 polymerase is provided. The expression host cells contain a copy of the T7 polymerase gene on their chromosome, which are under the control of *lacUV5*. Induction occurs through the addition of IPTG. More specifically, the pET32a(+) vector makes use of the T7*lac* promoter. This promoter contains a 25 bp *lac* operator sequence downstream of the promoter region. A *lac* repressor binds at this site which reduces transcription by T7 polymerase. Thus there are two mechanisms suppressing basal expression, at the T7 *lac* promoter and at the *lacUV5* promoter.

Alternatively, osmotic shock with NaCl can also be used for induction of expression using the pET32a(+) vector (Bhandari and Gowrishankar, 1997). In the specific strain (BL21+S) the T7 RNA polymerase has been placed under the control of the osmotically inducible *proU* promoter. NaCl induction also decreases the chances of expression of insoluble proteins.

The pET32a(+) vector adds three tags (Trx-tag, S-tag and His-tag) to the amino terminal of the target protein and an optional His-tag to the carboxy terminal. The His-tag added at the amino terminal can be used for affinity purification of the fusion protein. The S-tag added at the amino terminal can also be used for detection and purification. The S-tag sequence encodes a peptide (15 aa) that binds to the S-protein (a cleavage product of pancreatic ribonuclease A). The enzyme is reconstituted by the S-tag product and its activity can be measured. This tag can also be used for purification with affinity purification (immobilized S-protein which binds the S-tag very strongly). The Trx-tag (109 aa) at the amino terminal enhances the solubility of the target protein and catalyzes the formation of disulfide bonds in the cytoplasm of *trxB* mutants. All three amino terminal tags can be removed by the protease enterokinase, while thrombin can be used to remove the amino terminal His-tag and Trx-tag. The Origami(DE3) strain was chosen for expression of the target RVFV N protein in the pET32a(+) vector. This host strain has mutations in the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes. This helps with the solubility of the target protein. The plasmid map of pET32a(+) can be seen in Figure 32.

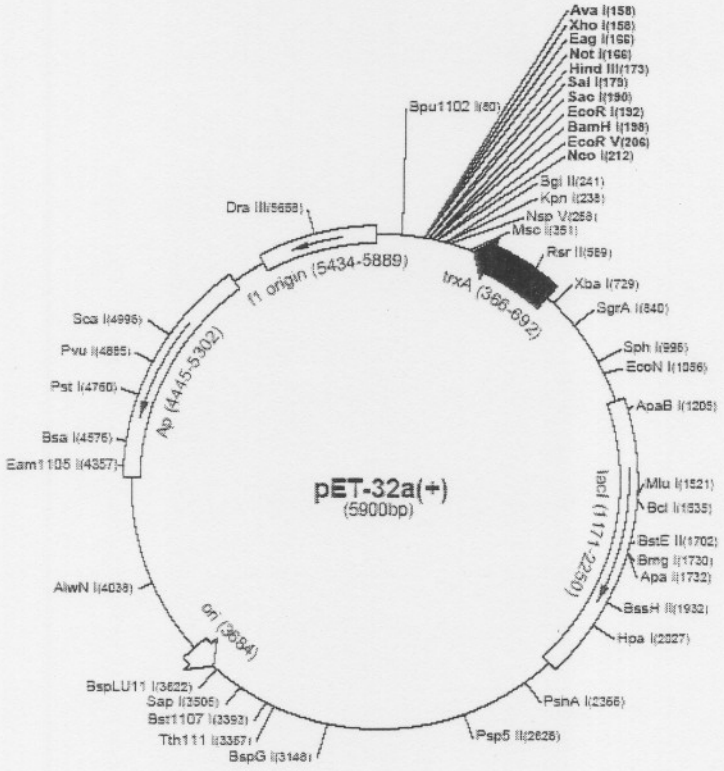


Figure 32. Plasmid map of pET32a(+)

4.2 Materials and Methods

4.2.1 Cloning into bacterial expression vectors:

Appropriate restriction enzymes (BamHI and XhoI) were identified for cloning the RVFV N gene into the bacterial expression vectors pET32a+ and pGEX4T-1. These vectors were prepared for cloning by double digestion with BamHI and XhoI (MATERIALS AND METHODS, 2.2.13), and subsequent gel-extraction (MATERIALS AND METHODS, 2.2.7). The RVFV N gene insert was prepared for cloning by double digestion with BamHI and XhoI of RT-PCR amplicon or recombinant plasmid containing the RVFV N gene insert (pRVFN-FBHTB), and subsequent gel-extraction of the correct fragment.

Ligation of the RVFV N gene into bacterial expression vectors, by means of compatible BamHI and XhoI 5'/3' overhangs, were based on the methods published in the literature (Sambrook and Russell, 2001) as described under MATERIALS AND METHODS (2.2.10). No insert DNA was added to the ligation control reactions. For cloning into pET32(a)+ (6.4 ng) different amounts of insert DNA (0 ng, 13.13 ng, 52.52 ng and 78.78 ng) was used. For cloning into pGEX4T-1 (16.4 ng) different amounts of insert DNA (0 ng, 13.13 ng, 52.52 ng and 78.78 ng) was used.

The resultant ligation mixtures were used to transform chemically competent SURE cells (Stratagene, catalogue number 200238) using the heat-shock method (MATERIALS AND METHODS, 2.2.11).

Uncut pET32a+ and pGEX4T-1 plasmid was used as transformation controls. A 100 µl volume of competent cells was transformed with 5 µl of each ligation reaction or uncut plasmid. Colonies containing recombinant plasmids were selected from LB agar plates containing the appropriate antibiotics (RESULTS, 4.3.1). Recombinant plasmids were collected (MATERIALS AND METHODS, 2.2.12) and analyzed by means of agarose gel electrophoresis, restriction site analysis with appropriate restriction enzymes and sequencing.

4.2.2 Bacterial expression of the RVFV Zim688/78 N protein using pRVFN-ET32 and IPTG induction:

The confirmed recombinant plasmid pRVFN-ET32 was used to transform competent Origami(DE3) cells (Novagen, Catalogue number 70630-4) according to the instructions of the manufacturer. The wild type pET32(a)+ plasmid containing no insert was also used to transform these cells to serve as a negative control for the expression. With regard to the scale of expression, generally for evaluating expression on small scale 5 ml cultures are induced; for antigen production 50 ml cultures are induced (10 x 5 ml). Colonies were picked from the LB-agar plates containing ampicillin and amplified overnight in 5 ml LB-broth containing 100 µg/ml carbenicillin and 10 µg/ml tetracycline. The overnight culture was diluted to an OD_{600} of 0.3 in 10 x 5 ml LB-broth containing 100 µg/ml carbenicillin and 10 µg/ml tetracycline, and incubated for 1 hour. When the OD_{600} reached 0.6, the expression inducer IPTG (Invitrogen, Catalogue number 15529-019) was added to a final concentration of 0.5 mM and the culture incubated for 4 hours at 37°C with shaking. A fraction of the cells were harvested at 3 hours after induction to determine solubility of the expressed proteins. The rest of the cells were harvested at 4 hours by centrifugation using a Beckman centrifuge (J2-21M, JA14 rotor) at 10 000 g, 4°C for 20 minutes. The supernatant was discarded and the cells resuspended in a mixture of the BugBuster® protein extraction reagent (Novagen, Catalogue number 70584-4), rLysozyme solution (Novagen, Catalogue number 71110-4) and Benzonase nuclease (Novagen, Catalogue number 70746-3) according to the instructions of the manufacturer, and incubated for 30 minutes at room temperature for lysis and protein extraction. The soluble phase proteins at 4h were collected by centrifugation in 1.5 ml Eppendorf tubes at 13 000 rpm in a bench-top centrifuge for 3 minutes. The soluble phase of each extract was collected and analysed with SDS-PAGE.

4.2.3 Purification of His-tagged proteins:

Recombinant RVFV fusion nucleoprotein containing a 6xHis tag at the N-terminal was purified from the soluble phase using a Protino® Ni-TED 1000 packed column (Machery-Nagel, Catalogue number 745110.50) according to the instructions of the manufacturer. Complete Mini, EDTA-free protease inhibitor (Roche, Catalogue number 11836170001) was added to the elution buffer. An aliquot of the purified protein was stored at 4°C, and the rest was stored at -70°C.

4.2.4 Bacterial expression using pRVFN-ET32 and NaCl induction:

The confirmed recombinant plasmid pRVFN-ET32 was used to transform chemically competent (Inoue, Nojima and Okayama, 1990) BL21+S cells (Bhandari and Gowrishankar, 1997), according to the procedure described by Inoue, Nojima and Okayama (1990). The wild type pET32(a)+ plasmid containing no insert was also used to transform these cells to serve as a negative control for the expression. Colonies were picked from the LB-agar plates containing ampicillin and amplified overnight in 5 ml LBON media (10 g/l tryptone, 5 g/l yeast extract diluted in distilled H_2O) containing 100 µg/ml carbenicillin. The overnight culture was diluted to an OD_{600} of 0.4 in a total volume of 5 ml LBON media containing 100 µg/ml carbenicillin, and incubated for 1 hour. When the OD_{600} reached 0.8, the expression inducer NaCl was added to a final concentration of 0.3M, and the culture incubated for 3 hours at 37°C with shaking (Bhandari and Gowrishankar, 1997). A volume of 500 µl of the induced cultures was collected every hour from before induction until 3 hours post-induction. The cells were harvested from these 500 µl collections in 1.5 ml Eppendorf tubes by centrifugation at 13 000 rpm for 1 minute using a bench-top Eppendorf centrifuge. The supernatant was discarded and the cells resuspended in a mixture of the BugBuster® protein extraction reagent (Novagen, Catalogue number 70584-4), rLysozyme solution (Novagen, Catalogue number 71110-4) and Benzonase nuclease (Novagen, Catalogue number 70746-3) according to the instructions of the manufacturer, and incubated for 30 minutes at room temperature for lysis and protein extraction. The soluble phase of each extract was collected and analysed with SDS-PAGE.

4.2.5 Bacterial expression using pRVFN-G4T1:

The confirmed recombinant plasmid pRVFN-G4T1 was used to transform chemically competent BL21 cells, as described by Inoue, Nojima and Okayama (1990). Colonies were picked from the overnight LB-agar plates containing ampicillin and amplified overnight in 2x YT media (1.6% Tryptone, 1% yeast extract and 0.5% NaCl, pH 7) containing 100 µg/ml carbenicillin. The overnight culture was diluted to an OD_{600} of 0.3 in 2x YT media containing 100 µg/ml carbenicillin, and incubated for 1 hour. When the OD_{600} reached 0.6, the expression inducer IPTG (Invitrogen, Catalogue number 15529-019) was added to a final concentration of 0.5 mM and the culture incubated for 3 hours at 37°C with shaking. A volume of 500 µl of the induced cultures was collected every hour from before induction until 3 hours post-induction. The cells were harvested from these collections by centrifugation at 13 000 rpm for 1 minute using a bench-top Eppendorf centrifuge. The supernatant was discarded and the cells resuspended in a mixture of the BugBuster® protein extraction reagent (Novagen, Catalogue number 70584-4), rLysozyme solution (Novagen, Catalogue number 71110-4) and Benzonase nuclease (Novagen, Catalogue number 70746-3) according to the instructions of the manufacturer, and incubated for 30 minutes at room temperature for lysis and protein extraction. The total fraction proteins of each extract was collected and analysed with SDS-PAGE.

4.3 Results

4.3.1 Cloning of RVFV N gene into bacterial expression vectors pET32(a)+ and pGEX4T-1:

The RVFV N gene with BamHI and XhoI restriction sites compatible for cloning was prepared as described in MATERIALS AND METHODS (3.2.1).

Generating cloning compatible vectors: BamHI and XhoI double digested bacterial vectors were also prepared using large-scale restriction enzyme digestions as described under MATERIALS AND METHODS (4.2.1). A total of 840 ng of pET32(a)+ and 786 ng pGEX4T-1 was double digested and purified by gel-extraction respectively. The yield of digested DNA after gel-extraction purification was approximately 160 ng of pET32(a)+ (3.2 ng/µl and $A_{260/280}$ of 1.6) and 410 ng of pGEX4T-1 (8.2 ng/µl and $A_{260/280}$ of 1.5). The yields of the different plasmids after double digestion and gel-extraction varied, probably because of variable amounts of DNA used in the digestion reactions and variability in the gel-extraction method (different gel slices etc.) According to the results, the DNA was purified sufficiently for further cloning.

The RVFV N gene was cloned into both bacterial expression vectors by means of ligation of their compatible BamHI and XhoI overhangs as described under MATERIALS AND METHODS (4.2.1).

The resultant ligation mixtures were used to transform chemically competent SURE cells.

Volumes of 100 µl and 200 µl of the transformation reactions with recombinant pET32(a)+ and pGEX4T-1 was plated on LB-agar plates containing ampicillin (100 µg/ml) and incubated overnight at 37°C. Several well isolated colonies were picked from these plates and used to inoculate 15 ml liquid LB media containing the same antibiotics for initial recombinant screening. All liquid media cultures were incubated overnight at 37°C shaking at 200 rpm.

A volume of 3 ml of each overnight culture was used for plasmid purification with the QIAprep Spin Miniprep Kit (QIAgen, Catalogue number 27106) as described under MATERIALS AND METHODS (2.2.12). The following names were given for the recombinant plasmids: pET32(a)+ → pRVFN-ET32 and pGEX4T-1 → pRVFN-G4T1. For initial recombinant screening, extracted plasmids from all picked colonies were analyzed by agarose gel-electrophoresis (results not shown). According to the migration patterns of plasmids from certain colonies on these gels, the colonies that were clearly bigger than the parent wild-type plasmid were subjected to further screening by restriction enzyme analysis. One colony of each recombinant plasmid (pRVFN-ET32 and pRVFN-G4T1) yielding the correct size fragments after digestions were selected and subjected to final restriction enzyme digestions to confirm the presence of the RVFV N gene in these recombinants. The RVFV N gene insert contains a restriction site for NdeI, while pGEX4T-1 lacks such a site. Therefore, linearization of pRVFN-G4T1 (5725 bp) with NdeI restriction enzyme could indicate the presence of the insert in the recombinant plasmids. Double digestion at the BamHI site and the asymmetric NdeI site should yield a fragment of specific size (454 bp) if the N gene insert is present in the recombinant plasmid. Double digestion of pRVFN-G4T1 with abovementioned enzymes should subsequently lead to one fragment

of 454 bp and a larger fragment corresponding to the remaining size of the specific recombinant plasmid (5271 bp). A volume of 5 µl of each digestion was analyzed by means of agarose gel-electrophoresis on 1.0% gels in TAE buffer (Sambrook and Russell, 2001), as can be seen in Figure 33.

From Figure 33 it can be seen that both pRVFN-G4T1 and pGEX4T-1 were linearized by digestion with BamHI (lanes 3 and 7). The linearized pGEX4T-1 (4969 bp) has also migrated a bit further through the gel than the linearized pRVFN-G4T1 which indicates that pRVFN-G4T1 is larger than pGEX4T-1, as would be expected when the 780 bp RVFV N gene insert is present in pRVFN-G4T1. Digestion of pRVFN-G4T1 with NdeI also yielded a partially linearized plasmid (lane 8), while pGEX4T-1 was not linearized by NdeI and still yielded supercoiled DNA (lane 4). From lane 8 it seems that NdeI did not totally linearize pRVFN-G4T1 since three bands are present, with one of the bands at the expected size (5725 bp). Double digestion of pRVFN-G4T1 with BamHI and NdeI yielded the expected 454 bp fragment (lane 9), further confirming the presence of the Zim688/78 RVFV N gene in the correct orientation for expression in pRVFN-G4T1. This band is very faint, probably due to low amount of DNA loaded on the gel. Double digestion of pGEX4T-1 with the same enzymes did not yield a 454 bp fragment.

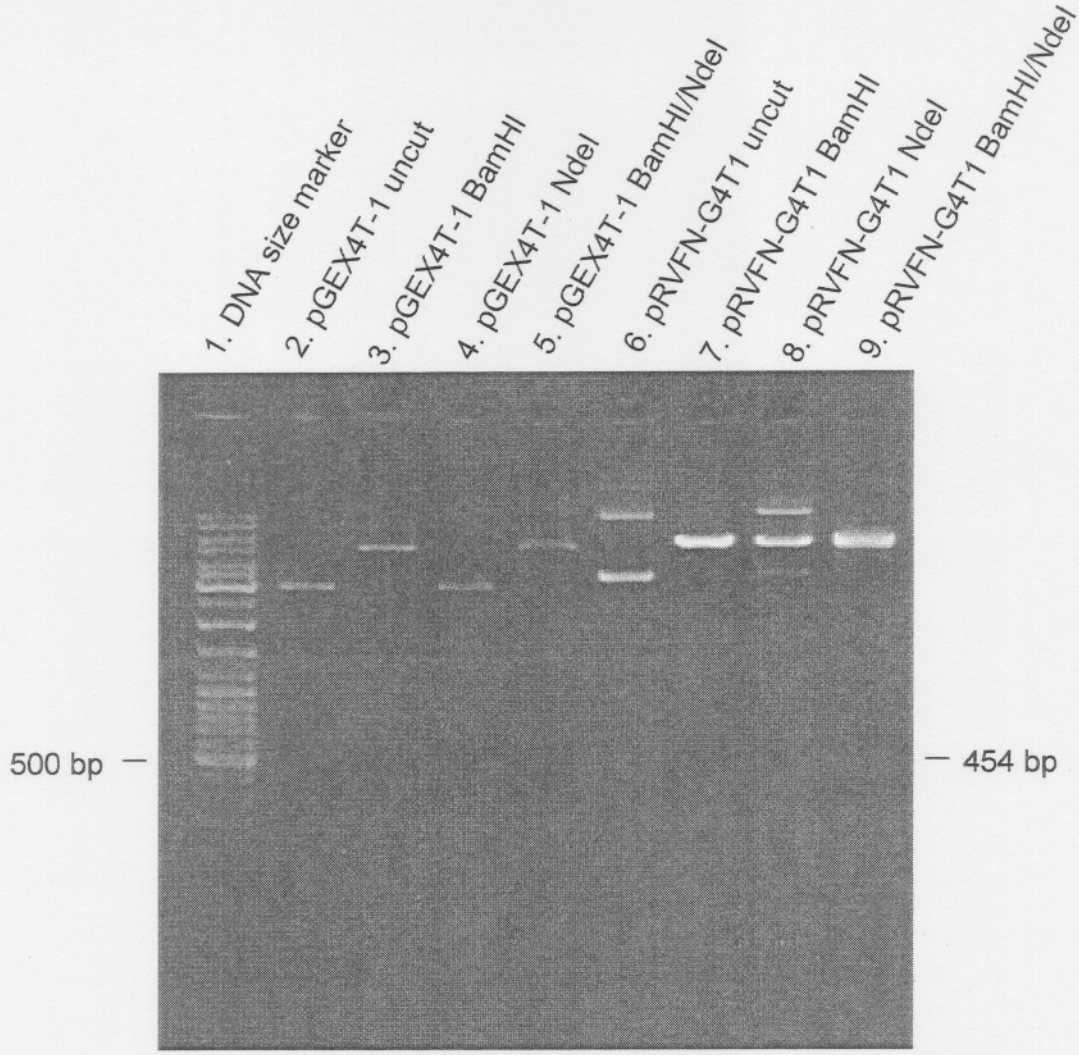


Figure 33. Agarose gel-electrophoretic analysis of restriction enzyme analysis of a plasmid obtained from the ligation of the RVFV N gene into pGEX4T-1 (pRVFN-G4T1). Lanes contain 5 µl of each sample: 1) O' GeneRuler DNA size marker (Fermentas, Catalogue number SM1173); 2) Uncut pGEX4T-1; 3) pGEX4T-1 digested with BamHI; 4) pGEX4T-1 digested with NdeI; 5) pGEX4T-1 digested with BamHI and NdeI; 6) pRVFN-G4T1 uncut; 7) pRVFN-G4T1 digested with BamHI, 8) pRVFN-G4T1 digested with NdeI and 9) pRVFN-G4T1 digested with BamHI and NdeI.

The wild type plasmid, pET32(a)+ has a site for HindIII. However, this site is lost when cloning into the BamHI/XhoI sites. Therefore, digestion of pET32(a)+ with HindIII and BamHI should lead to 25 bp and 5875 bp fragments, while digestion of pRVFN-ET32 with these enzymes should lead to 682 bp and 5958 bp fragments. A volume of 5 µl of each digestion was analyzed by means of agarose gel-electrophoresis on 1.0% gels in TAE buffer (Sambrook and Russell, 2001), as can be seen in Figure 34.

From Figure 34 it can be seen that both pET32(a)+ and pRVFN-ET32 was linearized by digestion with HindIII and BamHI (lanes 3, 4, 7 and 8). Double digestion of pET32(a)+ with BamHI and HindII yielded linearized DNA, but also an unexpected band at approximately 5000 bp (lane 5). Double digestion of pRVFN-ET32 with BamHI and HindIII yielded the expected result of a 682 bp fragment (lane 10).

To further confirm the correct cloning and reading frame of the Zim688/78 RVFV N gene insert into the bacterial expression vectors, sequence analysis was performed on the recombinant plasmid clones identified by agarose gel-electrophoresis and restriction enzyme analysis. The recombinants pRVFN-G4T1 and pRVFN-ET32 were sequenced with forward and reverse primers from both sides of the insert. These primers were synthesized and HPLC purified by the company Metabion. For pRVFN-G4T1 the primers were the 5'-pGEX primer (5' –gggCTggCAAgCCACgTTTggTg– 3') and the 3'-pGEX primer (5' –CCgggAgCTgCATgTgTCAgAgg– 3'). For pRVFN-ET32 the primers were the S·Tag primer (5' –CgAACgCCAgCACATggACA– 3') and the T7 terminator primer (5' –gCTAgTTATTgCTCAgCgg– 3'). All the sequencing results confirmed correct cloning of the N gene in both vectors (results not shown).

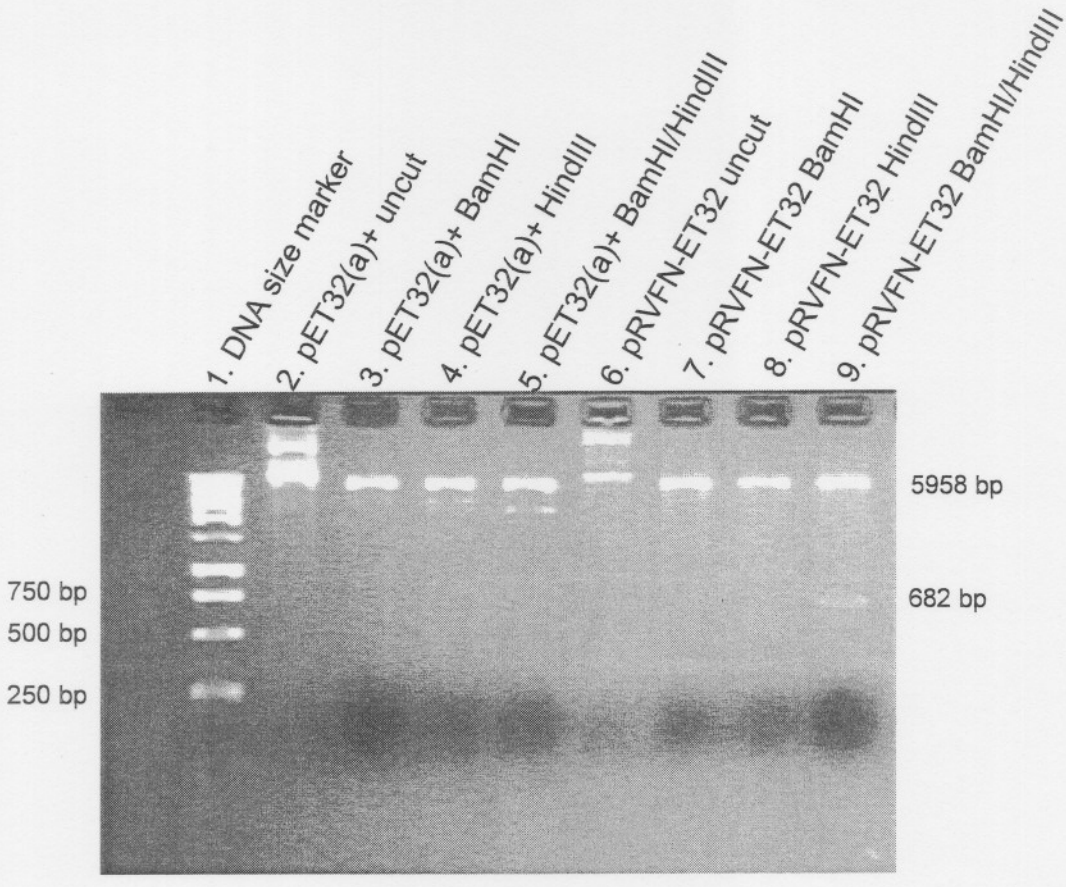


Figure 34. Agarose gel-electrophoretic analysis of restriction enzyme analysis of a plasmid obtained from the ligation of the gene encoding the RVFV N protein into pET32(a)+ (pRVFN-ET32). Lanes contain 5 µl of each sample: 1) 1kb DNA ladder (Promega); 2) Uncut pET32(a)+; 3) pET32(a)+ digested with BamHI; 4) pET32(a)+ digested with HindIII; 5) pET32(a)+ digested with BamHI and HindIII; 6) pRVFN-ET32 uncut; 7) pRVFN-ET32 digested with BamHI, 8) pRVFN-ET32 digested with HindIII and 9) pRVFN-ET32 digested with BamHI and HindIII.

4.3.2 Bacterial expression using pRVFN-ET32 and IPTG induction:

The confirmed recombinant plasmid pRVFN-ET32 was used to transform competent Origami(DE3) cells (Novagen, Catalogue number 70630-4) according to the instructions of the manufacturer. The wild type pET32(a)+ plasmid containing no insert was also used to transform these cells to serve as a negative control for the expression. Induction and expression was done as described in MATERIALS AND METHODS (4.2.2) for 4 hours. The cell pellet of the pRVFN-ET32 culture collected after 4 hours of induction weighed 0.4 g. The cells were lysed using the BugBuster protein extraction reagent with added Benzonase and rLysozyme (Novagen) as described under MATERIALS AND METHODS (4.2.2) for 30 minutes. Protein fractions were collected and stored. The total fraction of proteins, synthesized at 3 hours after induction of expression of colonies harbouring pRVFN-ET32, was analysed against the proteins in the soluble fraction at the same time using SDS-PAGE (MATERIALS AND METHODS, 3.2.8). The proteins were separated using SDS-PAGE with a 10% separating gel and a 3.9% stacking gel, as can be seen in Figure 35.

The expected size of the recombinant N protein with the added tags is 42 kDa (28 kDa N protein plus 14 kDa tags added by pET32a+). In Figure 35 it can be seen that the prominent band representing this fusion protein is at the expected size of 42 kDa, between the 37 kDa and 50 kDa bands of the molecular weight marker. From the figure it is clear that the over-expressed N protein constituted the majority of the proteins in the sample, as this band is very prominent. The recombinant N protein was also highly soluble as there is practically no visual difference between lane 1 (total fraction proteins) and lane 2 (soluble proteins). Because of the high expression levels and solubility of the recombinant N protein, the purification thereof was continued using metal chelate affinity chromatography.

Purification was done on the lysate collected at 4 hours after induction as described under MATERIALS AND METHODS (4.2.3) using a Ni column (Protino, Machery-Nagel) utilizing the 6x Histidine tag added to the N terminal of the recombinant protein by the pET32a(+) vector.

The soluble fraction of the extracted proteins of the pRVFN-ET32 expression at 4h, and the soluble fraction of the extracted proteins of the negative control (pET32a+) expression at 4h were analysed together with the His-purified RVFV rNucleoprotein from 4 hours using SDS-PAGE. The proteins were separated using SDS-PAGE separating gel of 10% and a stacking gel of 3.9%, as can be seen in figure 36.

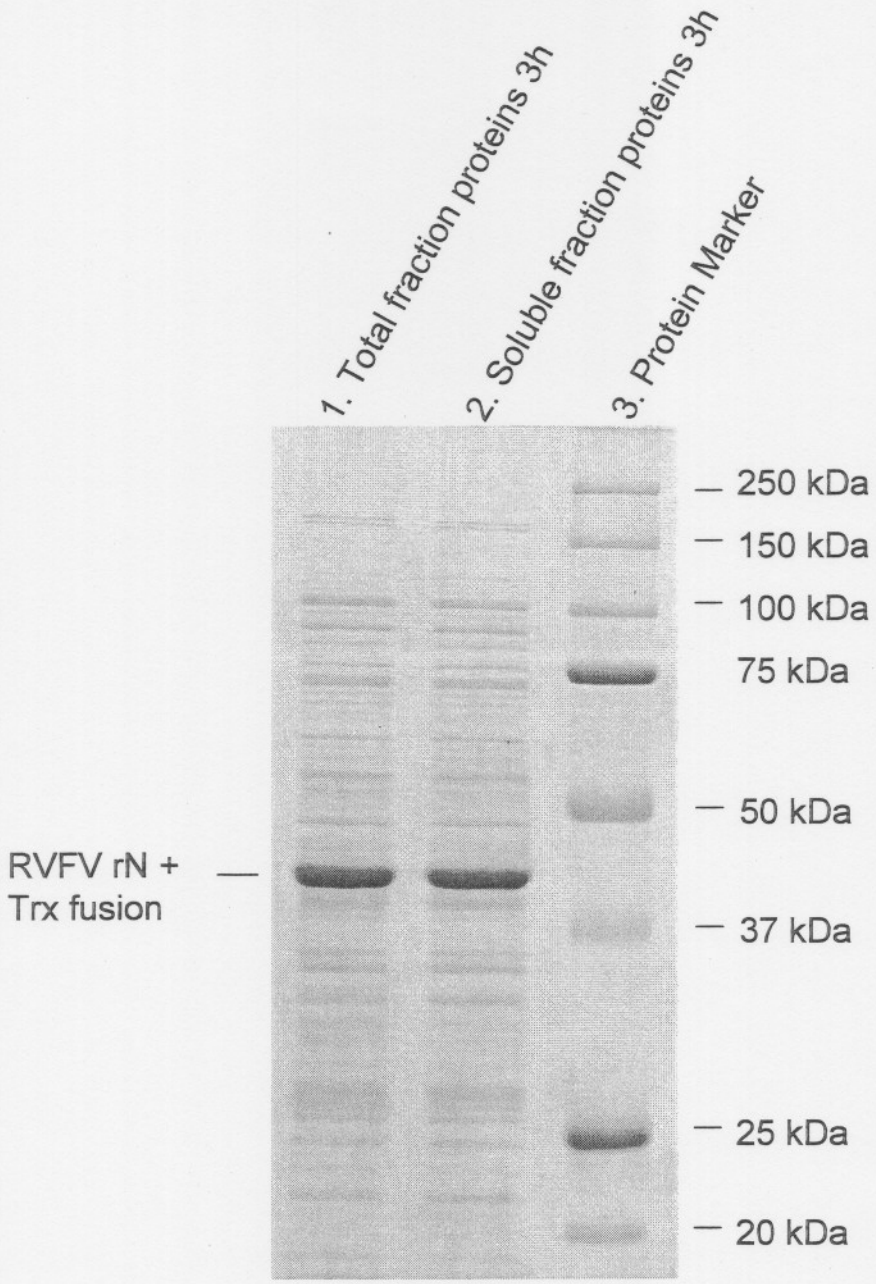


Figure 35. SDS-PAGE analysis of proteins at 3 hours post-induction expressed in Origami (DE3) cells containing the recombinant expression vector, pRVFN-ET32.

Lanes contain a 10 µl sample of 500 µl of each: 1) Total fraction proteins expressed after 3 hours of IPTG induction; 2) Soluble fraction proteins expressed after 3 hours of IPTG induction; 3) Protein molecular weight marker (BioRad,Catalogue number 161-0374)

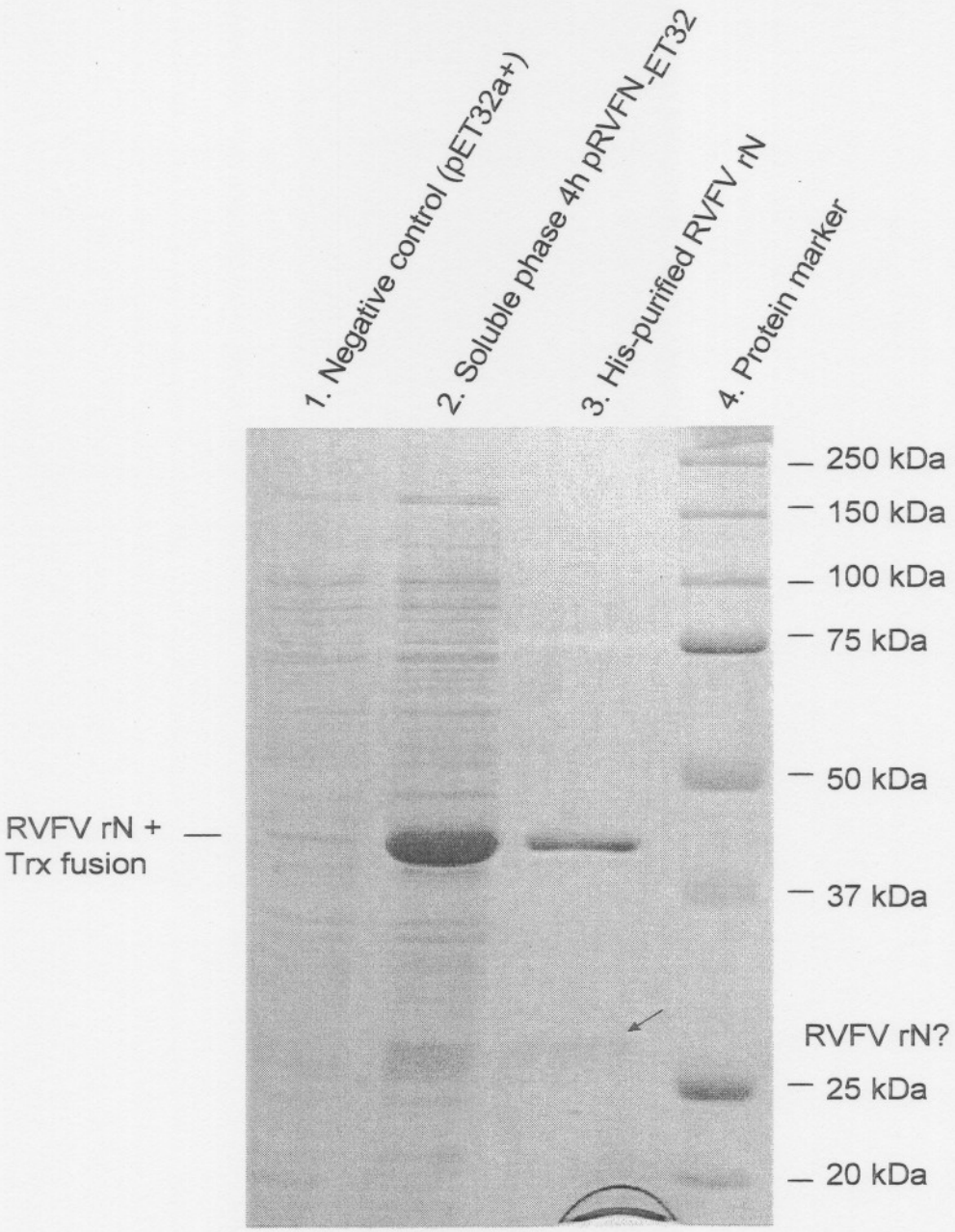


Figure 36. SDS-PAGE analysis of soluble proteins 4 hours post-induction expressed in Origami (DE3) cells containing the recombinant expression vector, pRVFN-ET32.

Lanes contain: 1) 10 µl of 500 µl of proteins expressed using wild type pET32(a)+ as negative expression control; 2) 10 µl of 2 ml of soluble fraction proteins expressed from pRVFN-ET32 after 4 hours of IPTG induction; 3) 10 µl of 4.5 ml of His-tag purified RVFV rN using a Protino Ni-TED column; 4) Protein molecular weight marker (BioRad,Catalogue number 161-0374). The arrow indicates a protein that co-purifies with the his-tagged rN.

4.3.3 Bacterial expression using pRVFN-ET32 and NaCl induction:

It was decided to evaluate the NaCl inducible expression system because of the low cost of NaCl compared to IPTG, the non-toxicity of NaCl compared to IPTG and because the literature states that the use of NaCl improves the chances of expressing a soluble recombinant protein (Bhandari and Gowrishankar, 1997). The confirmed recombinant plasmid pRVFN-ET32 was used to transform chemically competent NaCl inducible BL21+S cells (Bhandari and Gowrishankar, 1997), according to the procedure described by Inoue, Nojima and Okayama (1990). The wild type pET32(a)+ plasmid containing no insert was also used to transform these cells to serve as a negative control for the expression. Induction and expression was done as described in MATERIALS AND METOHDS (4.2.4). The soluble fraction of proteins at 1 hour intervals for 3 hours were analysed using SDS-PAGE. The proteins were separated using SDS-PAGE with a 10% separating gel and a 3.9% stacking gel, as can be seen in Figure 37.

The expected result of the expression of empty pET32a(+) vector was a band of the over-expressed 14 kDa tag. However this band is not clearly visible on the gel. This could be because the gel was over-run or because the expression did not work for unknown reasons. The expected result of the expression of pRVFN-ET32 was again a 42 kDa protein. In lanes 6 to 9 there is a band at the expected size of 42 kDa which increases in intensity over the 3 hour period. However, in lanes 1 to 4 (expression control) there is also a band at this size, but it does not increase in intensity over the induction time. This band can most probably be attributed to a cellular protein. Thus, from this figure one can see that the expression following NaCl induction did work, but expression levels was very low. With further optimization this system could produce higher levels of expression, but it was decided not to explore this option.

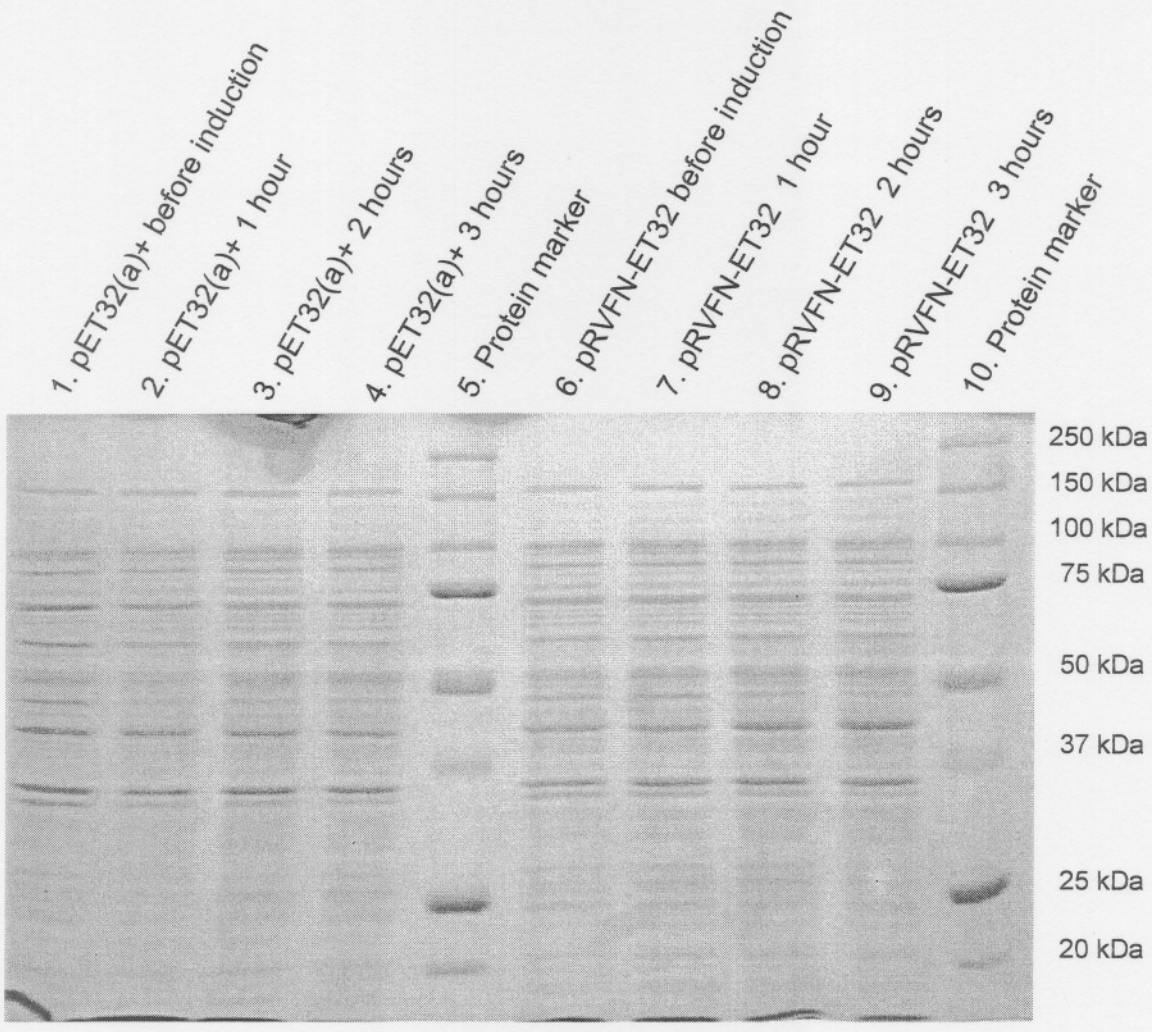


Figure 37. SDS-PAGE analysis of proteins expressed using the recombinant expression vector, pRVFN-ET32 in BL21+S cells.

Lanes contain 10 µl sample of 500 µl: 1) Proteins expressed using wild type pET32(a)+ as negative expression control before induction; 2) pET32(a)+ expression 1 h after induction; 3) pET32(a)+ expression 2 h after induction; 4) pET32(a)+ expression 3 h after induction 5) Protein molecular weight marker (BioRad,Catalogue number 161-0374); 6) Proteins expressed using recombinant pRVFN-ET32 before induction; 7) pRVFN-ET32 expression 1 h after induction;8) pRVFN-ET32 expression 2 h after induction;9) pRVFN-ET32 expression 3 h after induction;10) Protein molecular weight marker (BioRad,Catalogue number 161-0374).

4.3.4 Bacterial expression using pRVFN-G4T1:

The confirmed recombinant plasmid pRVFN-G4T1 was used to transform chemically competent BL21 cells, as described by Inoue, Nojima and Okayama (1990). Induction and expression was done as described in MATERIALS AND METHODS (4.2.5). The total fraction of proteins synthesized at 1 hour intervals for 3 hours after induction were analysed using SDS-PAGE. The proteins were separated using a 10% SDS-PAGE separating gel, as can be seen in Figure 38.

The expected size of the recombinant N protein plus the GST-tag added by the vector is 54 kDa (N protein 27 kDa and GST-tag 27 kDa). In Figure 38 it can be seen that there is a band at the expected size of 54 kDa, which increases in intensity from before induction to 3 hours post-induction. Thus, expression of the RVFV N protein was successful in the pGEX4T-1 vector. Before induction (lane 2) there is only a faint band at the position of the expected protein size. This could be attributed to an *E. coli* cellular protein or some leakage of the induction system leading to expression without induction. In lanes 3-5 it can be seen that the intensity of this particular band increases. Thus expression of the RVFV N protein + GST fusion increased over the three hours of induction. However, the lysates of these expression reactions were not clear, indicating that the expressed protein formed inclusion bodies and was therefore insoluble. The solubility of the protein can be improved by changing the induction conditions (increasing aeration, different inducer concentrations and lower temperatures). However, it was decided that it was not necessary to optimize this expression system further because the pET32(a)+ system produced higher expression levels and the RVFV N protein was soluble.

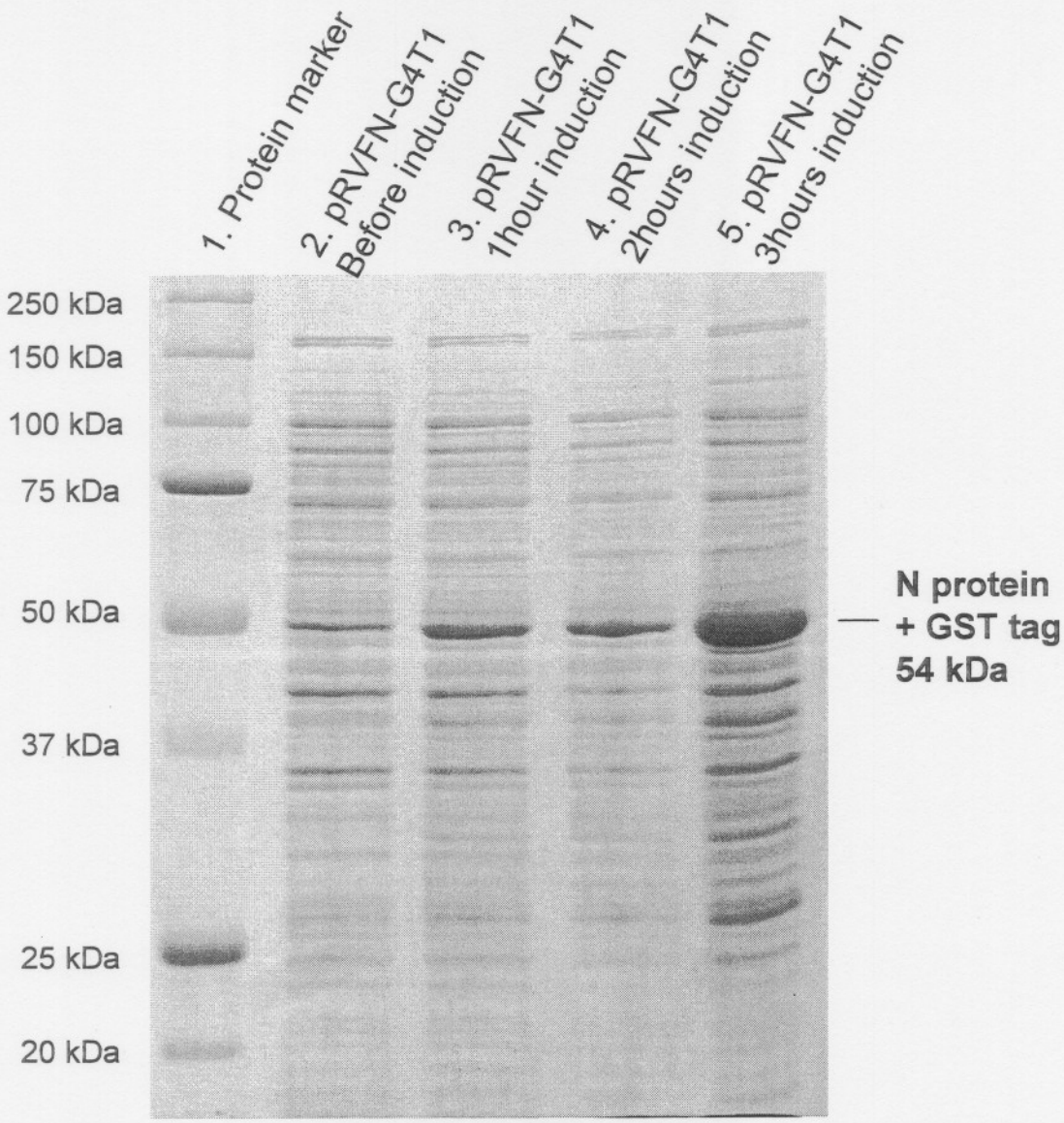


Figure 38. SDS-PAGE analysis of proteins expressed using the recombinant expression vector, pRVFN-G4T1.

1) Protein molecular weight marker (BioRad,Catalogue number 161-0374); 2) 8 µl of 500 µl of pRVFN-G4T1 expression before induction; 3) 7 µl of 500 µl of pRVFN-G4T1 expression 1 h after induction; 4) 6 µl of 500 µl of pRVFN-G4T1 expression 2 h after induction; 5) 5 µl of 500 µl of pRVFN-G4T1 expression 3 h after induction.

4.4 Summary

If it is at all feasible, bacterial expression of recombinant proteins is generally preferred to baculovirus expression in terms of the cost, ease and speed of the whole process. Once the gene of interest is cloned into the specific expression plasmid, expression can be done in three days at the most, whereas baculovirus expression could take weeks. *E. coli* cells are much easier to culture than insect cells, and its doubling time is muck quicker. There are various ways that can be exploited for induction of expression of the inserted gene, depending on the specific expression plasmid and the type of cells being used. Bacterial expression has been utilized in the past to express RVFV antigens. Keegan and Collet (1986) expressed portions of the G2 glycoprotein of RVFV in order to determine the amino acid sequences of antigenic determinants on the G2 protein.

The gene encoding the nucleoprotein of RVFV was cloned into two bacterial expression vectors. The expression vector pET32(a)+ makes use of the T7 expression system, thus the target gene is under control of the T7 promoter, which is IPTG inducible. In some strains of *E. coli* the T7 RNA polymerase is under control of the osmotic inducible *proU* promoter, which can be induced with osmotic shock with NaCl. The pET32(a)+ vector adds three tags to the recombinant protein; 6xHis, S-tag and Trx-tag. These tags can be used for detection and purification of the recombinant protein. The expression vector pGEX4T-1 utilizes the Glutathione S-transferase (GST) gene fusion system, and the target gene is under control of the *tac* promoter, which is IPTG inducible. A GST fusion tag is added to the N terminal of the recombinant protein which can be used for purification or detection of the recombinant protein. The RVFV N-protein was expressed using IPTG induction in Origami(DE3) cells (Novagen) and NaCl induction in BL21+S cells with the pET32(a)+ vector containing the RVFV N-gene.

Expression levels were very low in the NaCl inducible BL21+S cells using the recombinant pET32(a)+ vector (Figure 37). According to Bhandari and Gowrishankar (1997) the aim of using NaCl induction is to minimize inclusion body formation of the expressed protein, and thus lower the insolubility of the protein. According to the authors expression levels should be lower compared to IPTG induction, but the protein should be more soluble when using NaCl induction.

In contrast, in this study expression levels were very high after 3 and 4 hours of IPTG induction using the recombinant pET32(a)+ expression vector (Figure 35). The recombinant RVFV N protein was also mostly soluble. Usually proteins are less soluble in bacterial expression systems when using IPTG induction because of the lack of post-translational modification in bacterial cells. The toxicity of IPTG to the cells could also play a role in this. However, the pET32(a)+ expression vector adds a Trx-tag to the N terminal of the recombinant protein. This thioredoxin protein helps with disulfide bond formation in the target protein, which could lead to increased solubility. The solubility of the N protein in this expression system could most probably be attributed to this characteristic.

The recombinant protein expressed from the pET32(a)+ vector using IPTG induction was purified by affinity purification through a Ni column, utilizing the 6xHis residues added to the N terminal of the recombinant protein by the vector. The purification resulted in a very pure preparation of the fusion nucleoprotein (42 kDa), with only a very small amount of a single other protein (±27 kDa) very faintly

visible after SDS-PAGE analysis (Figure 36). Interestingly, the 27 kDa size of this second protein is identical to the size of the nucleoprotein without a fusion tag. The presence of this protein could be due to the fact that the N terminal primer (RVFVNPN3) for amplification of the N gene was designed to contain the ATG start codon of the N gene. Therefore, when this insert is cloned into an expression vector, translation could probably also start from this ATG and not only from the ATG of the complete tag added by pET32(a)+. Thus it is possible that the 27 kDa nucleoprotein without the pET32(a)+ fusion tag was also synthesized. However, this does not explain why it was co-purified with the fusion N protein, because the N protein itself is not histidine rich. A possible explanation is the ability of the N protein to oligomerize. According to Le May et al. (2005) the RVFV N protein can form dimers and other oligomers. Thus, it is possible that N protein dimers were purified and the dimers only denatured to form single polypeptides under the SDS-PAGE conditions. Thus, thus 27 kDa protein is probably not a contaminating protein, but, in fact, RVFV nucleoprotein without a fusion tag. Western blot analysis would be the way to confirm this.

Expression levels of rN were also high using the bacterial pGEX4T-1 vector with IPTG induction (Figure 38). However, the protein was largely insoluble. The insolubility could be attributed to a higher concentration of IPTG being used than recommended. One could possibly also optimize the growth rate of the cells during induction by lowering the temperature and decreasing aeration to increase solubility of the product.

Because of the high levels of expression and excellent purification of RVFV rN of Zim688/78 achieved with the bacterial pET32(a)+ vector and IPTG induction it was decided to use this recombinant protein and evaluate its potential as a diagnostic antigen in ELISAs. This is discussed in the next chapter.

CHAPTER 5

EVALUATION OF RECOMBINANT RVFV NUCLEOPROTEIN AS AN ANTIGEN FOR AN INDIRECT ELISA

5.1 Introduction

The classical methods for detection of antibodies against RVFV such as haemagglutination-inhibition, complement fixation, indirect immunofluorescence and virus neutralization are laborious, time consuming and pose a health risk to laboratory workers because they may potentially be exposed to the live virus. Therefore, the use of these methods is restricted to high biocontainment laboratories outside endemic regions. These methods are also unable to distinguish between different classes of immunoglobulins that enables one to determine if infection was recent (IgM antibodies usually occur only in the first weeks after infection) or historical (IgG antibodies can remain for many years after infection). There is an increasing demand for high-quality validated diagnostic tests for RVF to monitor vaccination status of laboratory staff, field veterinarians and livestock (Paweska *et al.*, 2005b).

Enzyme-linked immunosorbent assays (ELISA) are a rapid, sensitive methods with high specificity and sensitivity (Niklasson *et al.*, 1984). ELISAs are based on the ability of antigens or antibodies to attach to surfaces by passive adsorption, better known as coating. Most proteins can bind to plastic surfaces as a result of hydrophobic interactions between the protein and the plastic, and binding is dependent on the net charge of the protein. These antigens or antibodies interact with specific proteins in the test sample to form (immunocomplexes). These interactions are made visible by a detecting antibody labeled with an enzyme and the addition of substrate which results in a color development which can be measured (Crowther, 1995). Currently inactivated whole RVF virus is being used as antigen in ELISAs designed for antibody detection. This poses a health risk to the laboratory personnel who have to propagate the virus in tissue culture or animals. Production of antigen using infected cell-cultures is also a laborious and expensive process. There is, therefore, a need for a recombinant antigen that is easy to prepare, safe and cost-effective to produce. A test utilizing such an antigen should also be robust, accurate, repeatable and precise.

The ideal ELISA setup is an indirect ELISA (I-ELISA) because it uses the least steps of all the ELISAs and is therefore less time-consuming. This means quicker diagnosis and lower cost because of the saving on reagents. One limitation of the I-ELISA is that the antigen has to be very pure in order to be coated directly onto the immunoplates.

In this chapter experiments are described that were performed to evaluate the bacterially expressed and purified RVFV rN for purity and if it can effectively bind to the ELISA plate. There are many factors that could influence the outcome of an ELISA, these factors need to be optimized before starting with the validation of a specific test (Paweska *et al.*, 2003a). These factors include, for example, the type of ELISA plate being used. Different plates have different binding properties and will therefore bind antigens and antibodies better than the others. The coating buffer is also an

important factor. The difference in pH of various buffers could have a marked effect on the ELISA signal. The concentrations and dilutions of each antibody and antigen also need to be optimized for a new ELISA. This chapter will explain how the recombinant nucleoprotein-based I-ELISA was standardized and how applicable the recombinant N protein is as an antigen in different ELISA formats, especially I-ELISA.

5.2 Materials and Methods

Optimal dilutions of coating antigen adsorbed to the plate, reaction/control serum, enzyme-antibody conjugates and substrate solution were determined through checkerboard titrations (Crowther, 1995). The plates were washed with an Auto Plate Washer (ELx 405, BIO-TEK Instruments) and ELISA signals were read with a Universal Microplate Reader (ELx 800, BIO-TEK Instruments).

5.2.1 Sera

The control sera used for the ELISAs were derived from freeze-dried and gamma-irradiated stocks. Human control sera were prepared as described by Paweska *et al.* (2005b). Briefly: high positive control serum was prepared by pooling serum from 10 infected individuals from the 1998 RVFV outbreak in East Africa; negative control serum was prepared by pooling serum from 4 humans that had never been exposed to RVFV and had tested negative in serum neutralization and haemagglutination-inhibition tests. Low positive control serum was prepared by diluting strong positive serum in negative control serum. Livestock control sera were prepared as described by Paweska *et al.* (2003a). Briefly: ovine high-positive sera were collected from eight animals experimentally infected experimentally with the AR 20368 strain of RVFV. Negative sera were obtained by pooling together sera from six animals that had never been exposed to the virus and had tested negative in serum neutralization and haemagglutination-inhibition tests. Low-positive serum was obtained by diluting strong-positive serum with negative serum. Bovine high positive sera were obtained from ten naturally infected animals during the RVF outbreak in East Africa. Negative bovine sera were obtained by pooling sera from animals that had never been exposed to RVFV and that tested negative in serum neutralization and haemagglutination-inhibition tests. Low-positive bovine serum was prepared by diluting high-positive serum with negative serum.

5.2.2 Indirect ELISA for detection of IgG in humans and animals

The standard method for I-ELISA is given below. Several modifications and combinations of reagents and incubation periods were experimented with in order to optimize the ELISA protocol for the recombinant antigen. These included:

- Testing different plates with different binding characteristics from different manufacturers: MaxiSorp and PolySorp from Nunc and Costar plates.
- Testing two coating buffers with different pH: phosphate buffered saline (PBS pH 7.4) and carbonate-bicarbonate buffer (pH 9.6).
- Testing two coating techniques: one hour coating at 37°C in a humidified incubator and overnight coating at 4°C.
- Testing different horseradish peroxidase-conjugates (HRPO); a Protein G conjugate and specific anti-species conjugates.
- Titration of the antigen and the conjugates.

The standard protocol was as follows:

1. Coat MaxiSorp ELISA plate overnight at 4°C with 100 µl of RVFV antigen stock stored at 4°C in 1x PBS (pH 7.4). Dilution of stock antigen was different for specific ELISA applications
   Wash plate 3 times (Wash buffer 1x PBS pH7.4, 0.1% Tween20)
2. Block the plate for 1 hour at 37°C in a humidified incubator with 200 µl of 10% skim milk powder (Fluka, catalogue number 70166) diluted in 1x PBS
   Wash plate 3 times
3. Add 100 µl of 1:400 dilution of human or animal sera and controls (strong positive sera C++, low positive sera C+ and negative sera C-) in 2% skim milk and incubate for 1 hour at 37°C in a humidified incubator
   Wash plate 3 times
4. Add 100 µl of the appropriate HRPO-labeled anti-species specific antibody (detection system) diluted 1:5000 in 2% skim milk and incubate for 1 hour at 37°C in a humidified incubator
   Wash plate 3 times
5. Add 100 µl of HRPO ABTS-substrate [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] warmed to room temperature and incubate in the dark at room temperature for 30 minutes
6. Stop the reaction with 100 µl of 1x SDS
7. Read the OD at 405 nm

5.2.3 Indirect ELISA for detection of IgM in humans and animals

1. Coat MaxiSorp ELISA plate overnight at 4°C with 100 µl of RVFV antigen diluted in 1x PBS (pH 7.4)
   Wash plate 3 times (Wash buffer 1x PBS pH7.4, 0.1% Tween20)
2. Block the plate for 1 hour at 37°C in a humidified incubator with 200 µl of 10% skim milk powder (Fluka, 70166) diluted in 1x PBS
   Wash plate 3 times
3. Add 100 µl of 1:400 dilution of human or animal sera and controls (strong positive sera C++, low positive sera C+ and negative sera C-) in 2% skim milk and incubate for 1 hour at 37°C in a humidified incubator
   Wash plate 3 times

4. Add 100 µl of the appropriate HRPO-labeled anti-species specific antibody (detection system) at different dilutions in 2% skim milk and incubate for 1 hour at 37°C in a humidified incubator
   Wash plate 3 times
5. Add 100 µl of HRPO ABTS-substrate [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] warmed to room temperature and incubate in the dark at room temperature for 30 minutes
6. Stop the reaction with 100 µl of 1x SDS
7. Read the OD at 405 nm

5.2.4 IgM-capture ELISA for livestock

1. Coat a MaxiSorp ELISA plate overnight at 4°C with 100 µl rabbit anti-sheep IgM (µ-chain) antibody at a dilution of 1:500 in PBS (pH 7.4).
   Wash plate 3 times
2. Block the plate for 1 hour at 37°C in a humidified incubator with 200 µl of 10% skim milk powder diluted in 1x PBS
   Wash plate 3 times
3. Add 100 µl sheep anti-RVFV IgM control sera at a dilution of 1:400 dilution in 2% skim milk and incubate for 1 hour at 37°C in a humidified incubator, with duplicate strong positives (C++) in the top two rows, duplicate low positives (C+) in rows 3 and 4, negative sheep sera (C-) in rows 5 and 6 and milk containing no sera in rows 7 and 8
   Wash plate 3 times
4. Add 100 µl RVFV antigen diluted 1:200 in 2% skim milk and incubate for 1 hour at 37°C in a humidified incubator
   Wash plate 3 times
5. Add 100 µl mouse anti-RVFV antibody in a 1:1000 dilution in 2% skim milk and incubate for 1 hour at 37°C in a humidified incubator
   Wash plate 3 times
6. Add 100 µl goat anti-mouse IgG - HRPO conjugate diluted 1:8000 in 2% skim milk and incubate for 1 hour at 37°C in a humidified incubator
   Wash plate 3 times
7. Add 100 µl of HRPO ABTS-substrate [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] warmed to room temperature and incubate in the dark at room temperature for 30 minutes
8. Stop the reaction with 100 µl of 1x SDS
9. Read the OD at 405 nm

5.2.5 IgG-sandwich ELISA for sheep and goats

1. Coat a MaxiSorp ELISA plate overnight at 4°C with 100 µl of a 1:5000 dilution of mouse anti-RVFV antibody in PBS (pH7.4)
   Wash plate 3 times

2. Block the plate for 1 hour at 37°C in a humidified incubator with 200 µl of 10% skim milk powder diluted in 1x PBS
   Wash plate 3 times
10. Add 100 µl RVFV antigen diluted 1:400 in 2% skim milk and incubate for 1 hour at 37°C in a humidified incubator
    Wash plate 3 times
3. Add 100 µl sheep anti-RVFV IgG control sera at a dilution of 1:400 in 2% skim milk and incubate for 1 hour at 37°C in a humidified incubator, with duplicate strong positives (C++) in the top two rows, duplicate low positives (C+) in rows 3 and 4, negative sheep sera (C-) in rows 5 and 6 and milk containing no sera in rows 7 and 8
   Wash plate 3 times
4. Add 100 µl of a 1:8000 dilution of rabbit anti-sheep IgG + HRP conjugate in 2% skim milk and incubate for 1 hour at 37°C in a humidified incubator
   Wash plate 3 times
5. Add 100 µl of HRPO ABTS-substrate [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] warmed to room temperature and incubate in the dark at room temperature for 30 minutes
6. Stop the reaction with 100 µl of 1x SDS
7. Read the OD at 405 nm

5.2.6 Statistical calculations:

Positive to negative signal ratio:

$$Pos/Neg = OD_{C++}/OD_{C-}$$

Percentage positivity (PP):

PP = (Average OD value of duplicate serum sample/average of intermediate C++ values) x 100

where OD C++ is the OD reading from the strong-positive control and OD C- is the OD reading from the negative control.

5.3 Results

5.3.1 Optimization of ELISAs based on *E. coli* expressed RVFV rN antigen for detection of anti-RVFV antibodies using control serum panels

As mentioned previously the ideal ELISA setup for detection of antibodies is the indirect ELISA because of its speed and lower cost. It was therefore decided to optimize an indirect ELISA using the recombinant antigen derived from the pRVFN-ET32 construct as the coating antigen. The layout for the I-ELISA is shown in Figure 39.

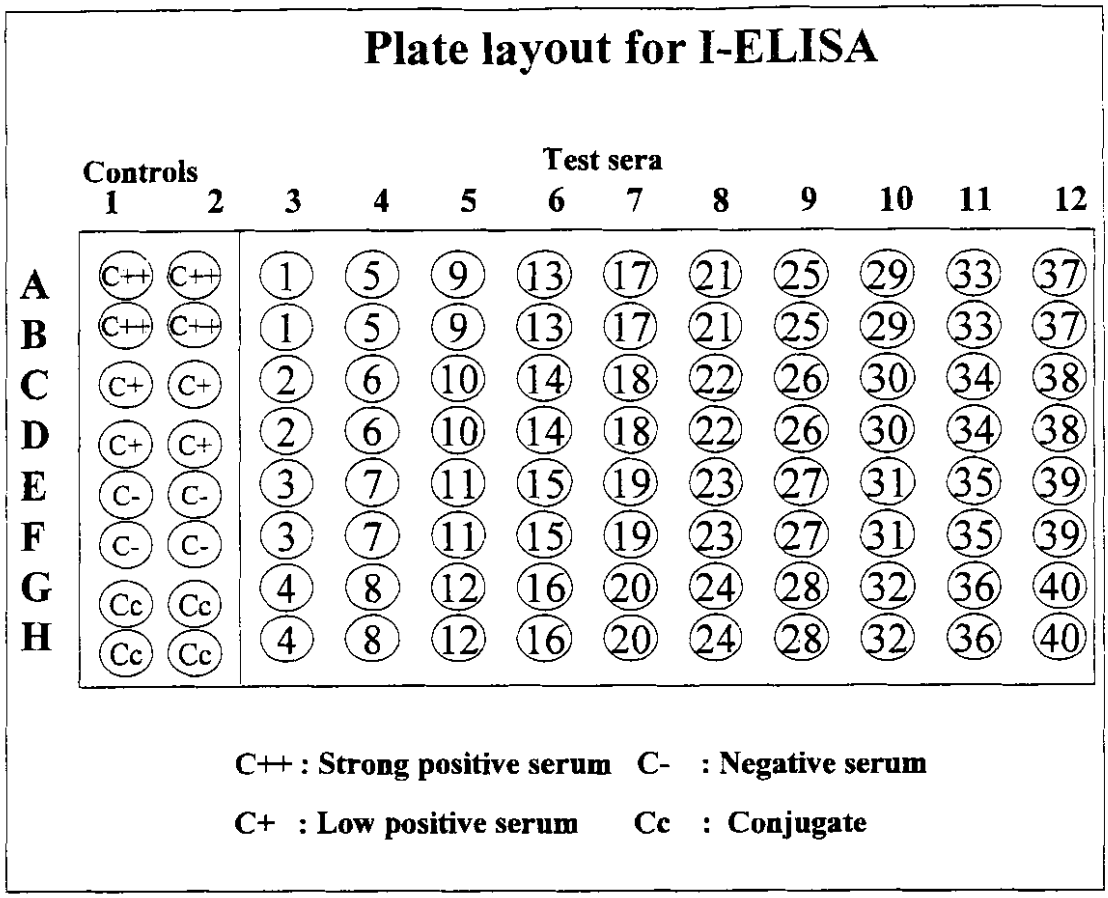


Figure 39. Plate layout for indirect ELISA.

Different ELISA plates, coating procedures, coating buffers, HRPO conjugated antibodies and concentrations of antibodies and antigens were experimented with. Three different kinds of plates (Polysorp, Maxisorp and Costar) either coated for 1 hour at 37°C or overnight at 4°C were compared. The I-ELISA, and calculations of the positive to negative signal ratios, were done using human control serum as described in the section MATERIALS AND METHODS (5.2). The level of positive to negative signal ratio achieved by a certain ELISA using controls is an indication of the performance of the ELISA. A ratio higher than 1:10 generally indicates that the test has the ability to clearly differentiate between sera containing various levels of antibodies. Two-fold dilutions from 1:50 to 1:102400 of the recombinant antigen were used for coating to establish its optimal dilution for routine runs of the I-ELISA. The results for the indirect human IgG ELISA using three different plates and 1 hour coating at 37°C are shown in Figure 40.

Figure 40 was drawn from the raw data given in Table 12 (Appendix 1) and demonstrates that the Maxisorp plate outperforms the other two ELISA plates, having a higher positive to negative signal ratio over the full range of dilutions (Figure 40). The line of the Maxisorp plate crosses the 10 pos/neg line at the dilution of the antigen between 2.9 and 3.2 $\log_{10}$ whereas the other two plates at the antigen dilution between 2.6 and 2.9 $\log_{10}$ dilution. This indicates that the antigen can be used at a higher dilution with Maxisorp plates than with Costar and Polysorp plates. The purified antigen titration curve had the characteristic slope as expected for biological material. One can see that the curve starts at a low point, increases to a peak and turns and decreases again. This means that there is higher unspecific background with negative control serum at relatively low dilutions than at higher dilutions of the antigen (log10 1.7 – 2). This nonspecific background at the lower dilutions can most probably be

attributed to *E. coli* cellular proteins that are detected as antigens in the control sera. However, at the higher dilutions of the RVFV nucleoprotein antigen the background becomes lower, the pos/neg ratio increases to a certain point, and then decreases with further antigen dilution. The potential role of working dilution of conjugate was also optimized in a later experiment (Figure 46).

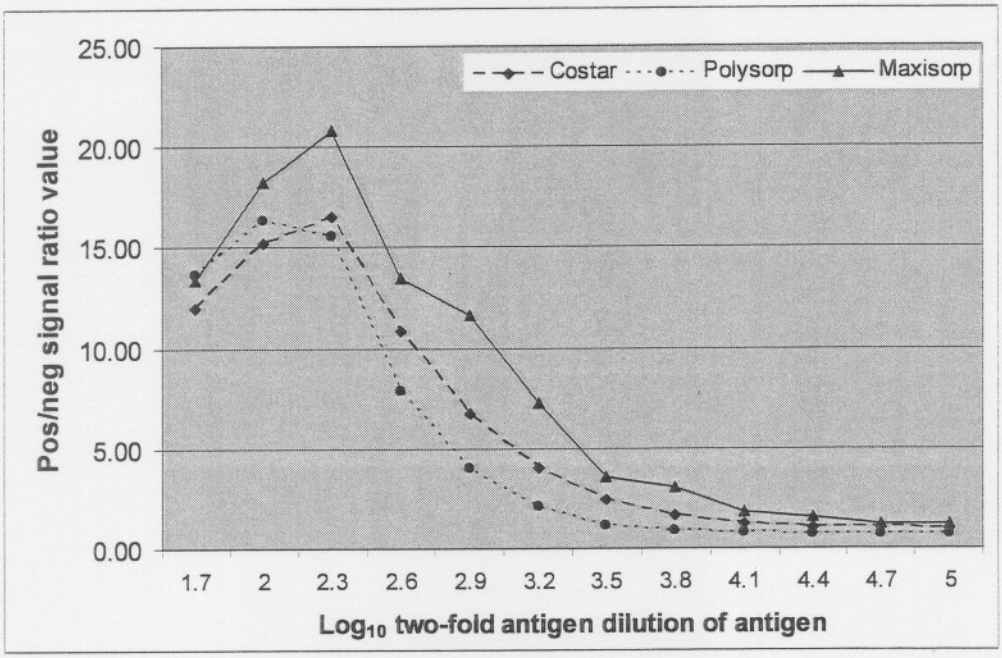


Figure 40. Positive to negative signal ratios of the indirect ELISA using three different ELISA plates with 1 hour coating at 37°C. Costar plate (♦), Polysorp (●) and Maxisorp (▲).

The performance of these ELISA plates was also evaluated using overnight coating at 4°C. The results from this experiment are shown in Figure 41, which was drawn from the raw data given in Table 13 (Appendix 1). From Figure 41 it can be seen that the Maxisorp plate outperforms the other two ELISA plates, having a higher positive to negative signal ratio at all dilutions used. The line of the Maxisorp plate crosses the 10 pos/neg ratio level at a higher dilution of the antigen (between the 3.2 and 3.5 $\log_{10}$ dilution of the antigen) than the other two plates (between the 2.9 and 3.2 $\log_{10}$ dilution). These results show that overnight coating at 4°C allows for higher antigen dilution compared to 1 hour coating at 37°C. This is probably due to more efficient binding of the antigen and confirmed that in comparison to Costar and PolySorp, the MaxiSorp immunoplates provide higher pos/neg signal ratio at different incubation temperatures over a range of different antigen dilutions. The purified antigen titration curve had the same slope characteristics as shown in Figure 41.

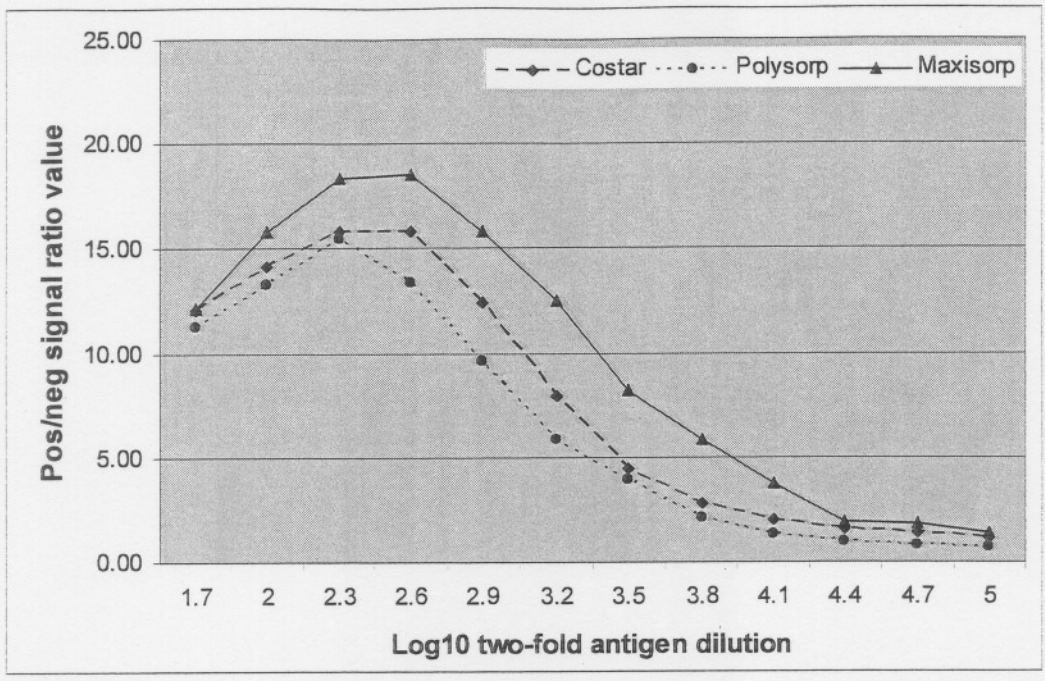


Figure 41. Positive to negative signal ratios of the indirect ELISA on three different ELISA plates coated overnight at 4°C. Costar (♦), Polysorp (●) and Maxisorp (▲).

Because proteins have different charges at different pH values, it was decided to investigate this aspect of assay optimization was assessed with two different coating buffers namely PBS at pH 7.4 and carbonate/bicarbonate buffer at pH 9.6. The results of the resulting I-ELISAs are shown in Figure 42.

Figure 42 was drawn from the raw data given in Table 14 (Appendix 1). It clearly shows that the carbonate/bicarbonate buffer outperforms the PBS buffer at all dilutions. The positive to negative signal ratio for the I-ELISA is higher when using carbonate/bicarbonate buffer compared to PBS buffer. Possible explanations may be that the conformation of the antigenic allows for better presentation at pH 9.6 or that this pH allows for better binding of the recombinant nucleoprotein to the plate. The carbonate/bicarbonate line crosses the pos/neg signal ratio 10 line at a higher dilution of the antigen than with PBS. This means that the antigen can be used at a higher dilution while still giving acceptable OD readings. Based on these results it was decided to use a 1:2000 dilution of the antigen for coating. Titration of the purified antigen resulted in the same curve as shown in Figure 42.

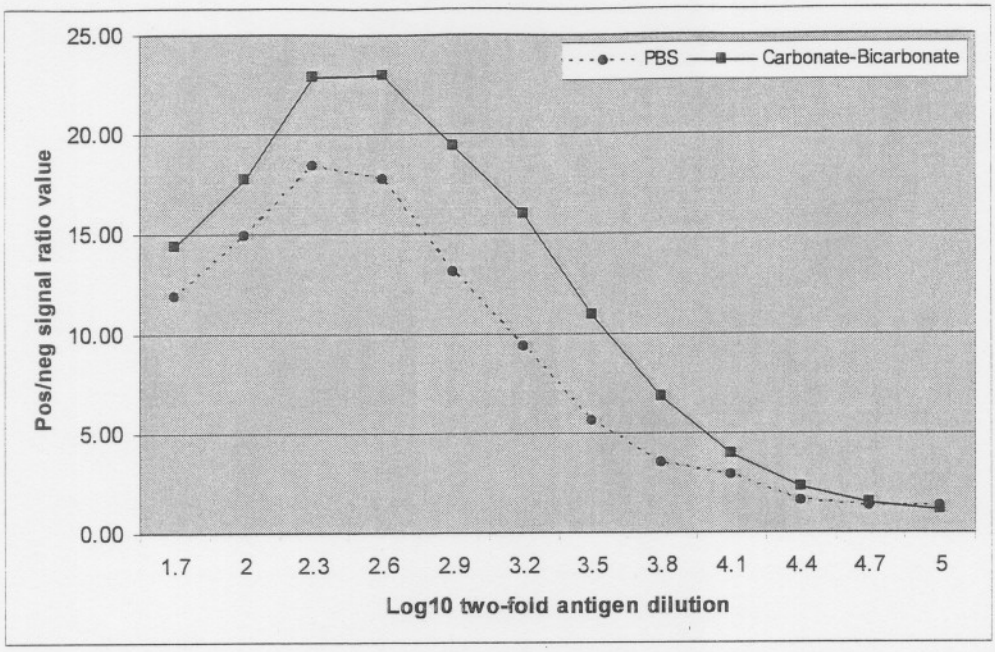


Figure 42. Positive to negative signal ratios of the indirect ELISA on Maxisorp plate coated overnight at 4°C using two different coating buffers. PBS coating buffer (♦) and carbonate/ibcarbonate buffer (■).

To determine the importance of antigen purity an ELISA run under the forementioned conditions (overnight coating of MaxiSorp plates and Carbonate/Bicarbonate buffer), was done using unpurified antigen, purified antigen and *E. coli* cellular proteins without RVFV rN. Each antigen was tested with strong positive and negative control sera. The results of this experiment are shown in Figure 43 which was drawn from the raw data given in Table 15 (Appendix 1). The titration curve of the purified antigen is similar to that of the previous experiments and shows a much better result compared to the unpurified antigen. The nonspecific background with the unpurified antigen was much higher than with the purified antigen. This is most probably due to the *E. coli* proteins present in the unpurified antigen. Most humans and animals have antibodies against *E. coli.* Therefore, the nonspecific background is likely due to the presence of antibodies against *E. coli* in the control sera which detects the bacterium's cellular antigens. That this is the case is clear from comparing the curve of the *E. coli* background with the antigen control curve (no antigen used). If no antibodies could be detected, the curves should have coincided. However, the curve of the *E. coli* background is above the curve of the negative control (Figure 43). Titration of the purified antigen resulted in the same curve as shown in Figure 42.

In conclusion the purified antigen gives minimal background activity with the negative control serum and the pos/neg signal ratio indicates that the antigen was sufficiently purified for further ELISA standardization.

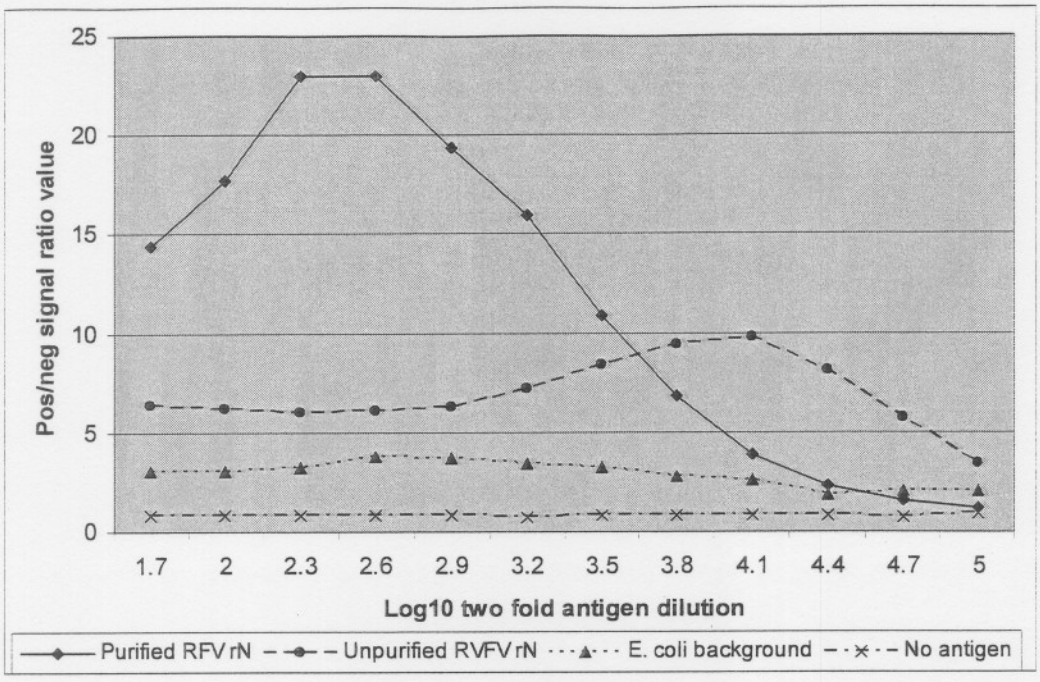


Figure 43. rN RVFV positive to negative signal ratio of the indirect ELISA on Maxisorp plate with purified and unpurified rN RVFV antigen coated overnight at 4°C using carbonate/bicarbonate buffer. Purified antigen (♦), unpurified antigen (●), *E. coli* proteins as antigen (▲) and the antigen control (×).

The ability of the recombinant nucleoprotein antigen to detect antibodies in cattle and sheep was first evaluated using a panel of standardized control sera obtained from the SPU-NICD. Detection of anti-RVFV antibodies in control serum from sheep and cattle was compared to detection in humans using an optimized protocol for each control serum set. The nett OD_{405} values for each control serum of human, sheep and cattle are shown in Figure 44.

Figure 44 was drawn from the raw data given in Table 16 (Appendix 1). From Figure 44 it can be seen that the high positive control serum from each species gave a high signal that is easily distinguishable from low positive and negative control sera. There is a slightly higher non-specific background in the bovine negative control serum, corresponding with the literature stating that ruminant sera generally have a relatively high background (Paweska *et al.* 2003a).

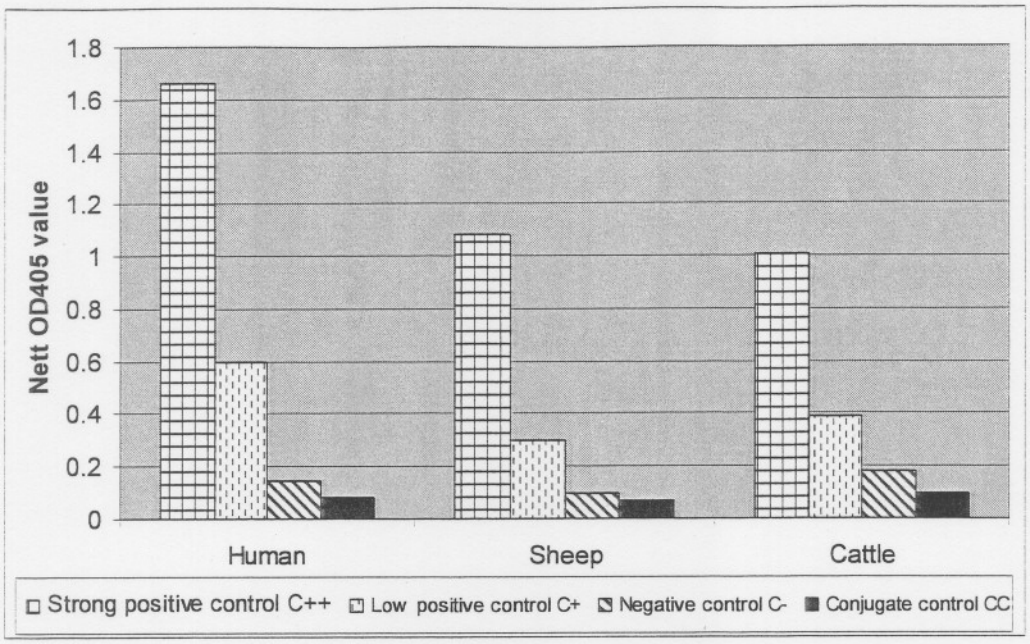


Figure 44. Nett OD_{405} values of human, sheep and cattle serum controls in an I-ELISA using RVFV rN as antigen and specific anti-species conjugates.

Protein G binds to a specific binding site on immunoglobulin G, the Fc region. This protein can be conjugated and thus used for the detection of any IgG in a serum sample. The advantage of optimizing a test with this protein is that the same test can be used for the detection of IgG antibodies in humans and animals. Therefore, the performance of this protein was also evaluated in the I-ELISA for detection of antibodies in humans, sheep and cattle sera. A Maxisorp ELISA plate was coated with a 1:2000 dilution of the recombinant RVFV nucleoprotein antigen. The same human, sheep and bovine control serum set was used as described above. A titration of Protein G from 1:1000 to 1:8000 was carried out and pos/neg ratios were calculated and plotted on the graph shown in Figure 45.

Figure 45 was drawn from the raw data given in Table 17 (Appendix 1). Figure 45 shows that the I-ELISA with Protein G gave different results in the three species tested, however the high positive control serum from each species gave a signal that is easily distinguishable from low positive and negative control sera (Figure 45).

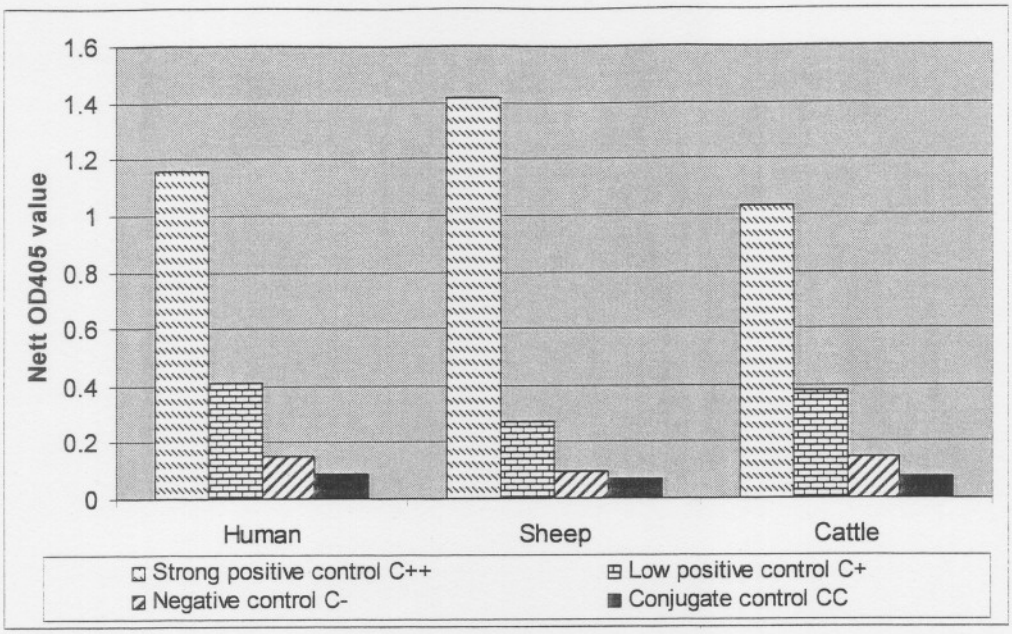


Figure 45. Nett OD_{405} values of human, sheep and cattle control sera in I-ELISA using RVFV rN as antigen and Protein G. conjugate.

It is important to be able to detect IgM antibodies in humans and animals because the presence of IgM antibodies indicates that the RVFV infection with the virus was recent. Therefore, an experiment was done to evaluate the applicability of the rN RVFV antigen for an indirect IgM ELISA. The I-ELISA for the detection of IgM antibodies against RVFV in animals was done as described under Materials and Methods. Three dilutions of the antigen (1:500, 1:1000 and 1:2000) were tested against different serial two-fold dilutions of the HRPO conjugate (1:500 to 1:4000). The results for this ELISA are shown in Figure 46.

Figure 46 was drawn from the raw data given in Table 27 (Appendix 1). All three dilutions of the antigen yielded titration curves with the expected decreasing tendency (Figure 46). The curve of the 1:500 dilution of the antigen is above the curves of the 1:1000 and 1:2000 dilutions, which is also expected as a higher concentration of the antigen would cause a higher ELISA signal. The best results were obtained when using a 1:500 dilution of the antigen. All three curves do, however, cross the positive negative ratio of 10. However, one can see that this is due to a very low background and not necessarily to a strong signal given by the strong positive control serum (C++) (Table 27). It was therefore decided to use 1:500 dilutions of both antigen and HRPO conjugated antibody for the indirect IgM ELISA in sheep. This experiment confirmed that the rN RVFV antigen can, in principle, detect IgM antibodies against RVFV in standardized positive control serum known to be positive for IgM.

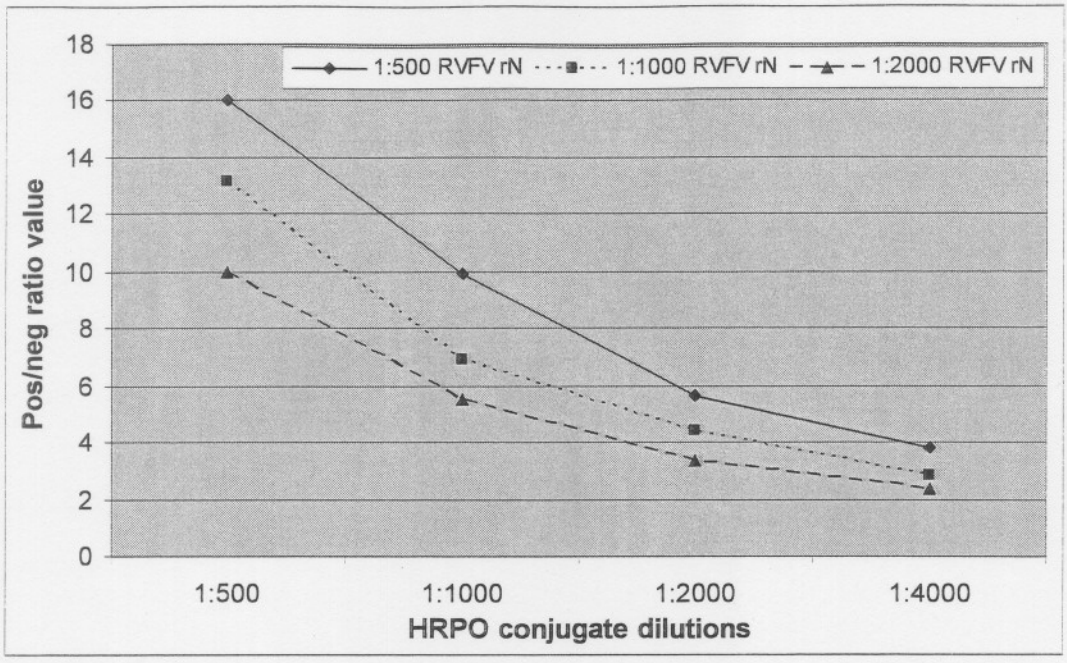


Figure 46. Indirect IgM ELISA for the detection of IgM antibodies against RVFV in sheep, using three dilutions of the recombinant RVFV nucleoprotein antigen against various dilutions of the HRPO conjugated detecting antibody. The 1:500 dilution of the antigen (♦), 1:1000 dilution (■) and 1:2000 dilution (▲).

A pilot experiment was done to test the applicability of the recombinant nucleoprotein antigen in an IgG sandwich ELISA for the detection of IgG in sheep and goats as described in Materials and Methods. The recombinant antigen was titrated from 1:1000 to 1:8000 on the plate (each dilution tested in triplicate). The average OD_{405} values were determined for each dilution of the antigen (Figure 47).

Figure 47 was drawn from the raw data given in Table 28 (Appendix 1). Figure 47 shows how OD value changes with decreasing antigen titre. There was a clear distinction between positive and negative controls. Thus, in principle, the recombinant RVFV N antigen can be used for the sandwich ELISA to detect IgG in animals.

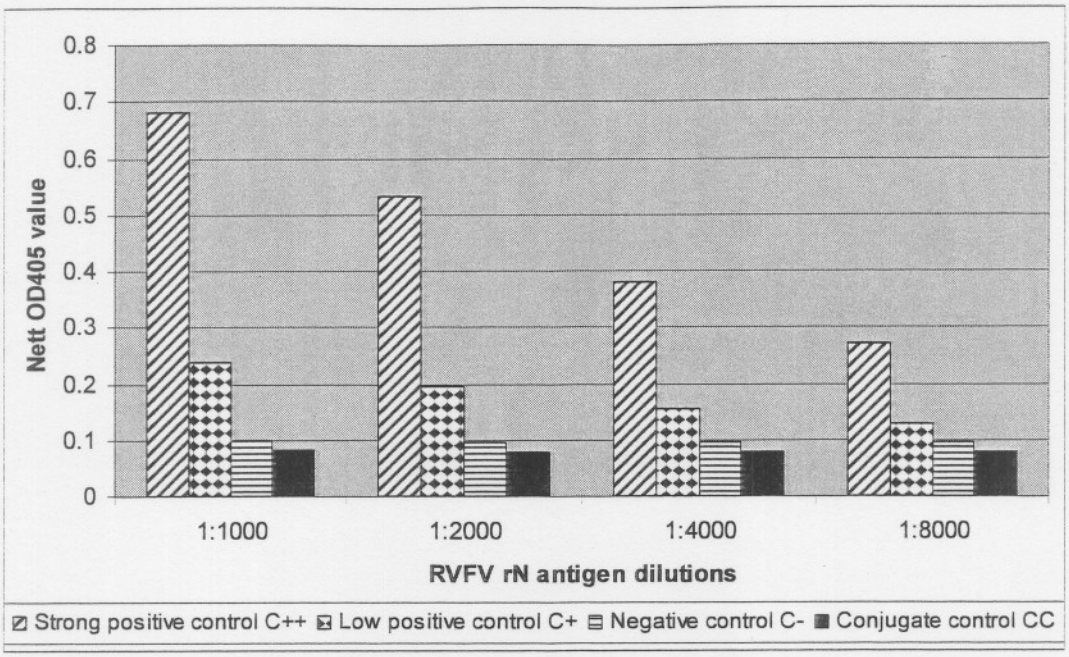


Figure 47. Titration of rN RVFV antigen in a sandwich ELISA for the detection of IgG antibodies against RVFV in a panel of sheep sera.

In another pilot experiment the applicability of the recombinant nucleoprotein antigen was tested for use in a capture ELISA for the detection of IgM in livestock as described in Materials and Methods. The recombinant antigen was titrated from 1:1000 to 1:8000 on the plate (each tested in triplicate) as shown in Figure 48.

Figure 48 was drawn from the raw data given in Table 29 (Appendix 1). Figure 48 shows the tendency for OD value to decrease with the progressive dilution of the antigen. There was a clear distinction between positive and negative controls. Thus, in principle the recombinant RVFV N antigen can be used in an IgM capture ELISA for IgM detection in animals.

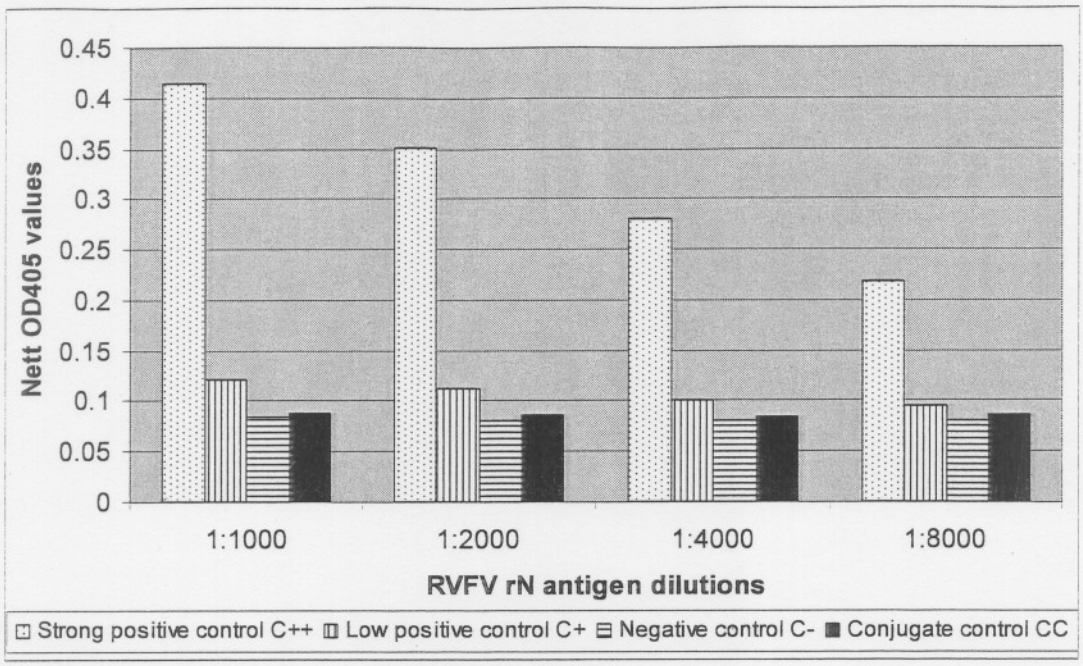


Figure 48. Titration of rN RVFV antigen in a capture ELISA for the detection of IgM antibodies against RVFV in a panel of sheep control sera.

5.3.2 Evaluation of the recombinant RVFV nucleoprotein expressed in *E. coli* as an antigen in the I-ELISA for detection of anti-RVFV antibodies in human patient sera:

To show the applicability of the recombinant antigen for detection of antibodies in field sera, sera of eight RVFV patients were tested. The sera were known to be positive for IgG antibodies with VN titres of 1:10 to 1:1280.

Figure 49 was drawn from the data given in Table 47 and 48 (Appendix 1). From Figure 49 it can be seen that the 5 sera from vaccinated humans were highly positive (between 60 and 100 PP) whilte the remaining 3 were low positive (between 20 and 40 PP). The titration curves of all 8 sera gave the expected result of PP decreasing with dilution of IgG antibodies.

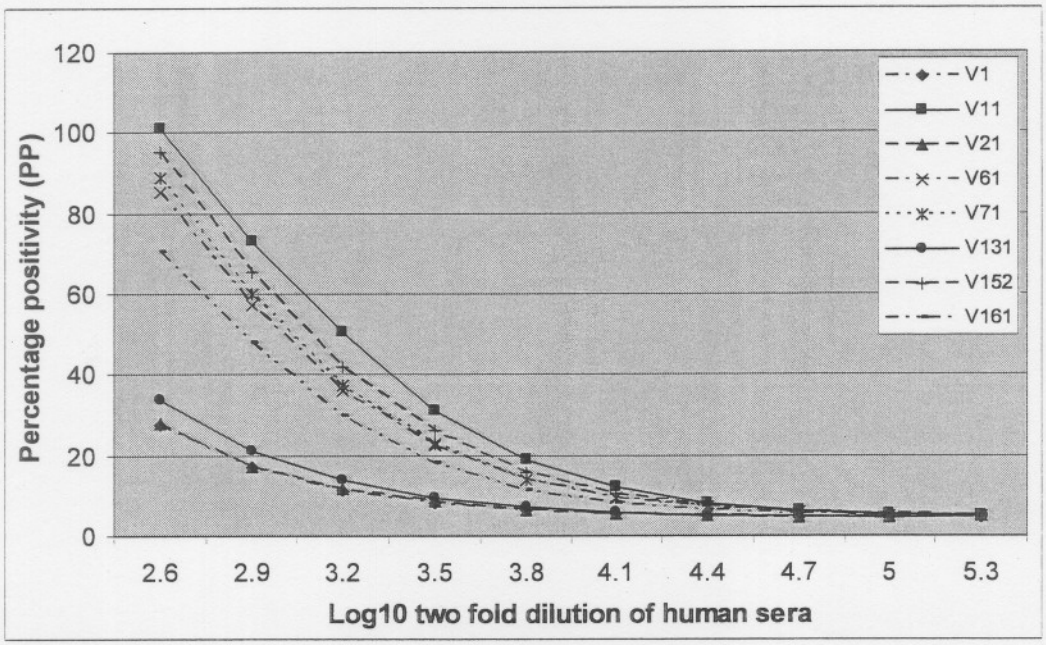


Figure 49. Titration of eight sera from RVF patients by I-ELISA using RVFV rN for detection of IgG antibodies. The symbol for each serum on the graph is displayed in the legend in the figure.

Furthermore, another eight human sera from patients infected with RVFV in 1998 in Kenya were titrated from 1:400 to 1:204800 in two-fold dilutions and tested for IgM antibodies.

Figure 50 was drawn from the data given in Table 49 and 50 (Appendix 1). From Figure 50 it can be seen that 7 of the infected human sera were highly positive (between 50 and 250 PP) and 1 was low positive (below 50 PP). The titration curves of all 8 sera gave the expected result of PP decreasing with dilution, representing decreasing amount of IgM antibodies.

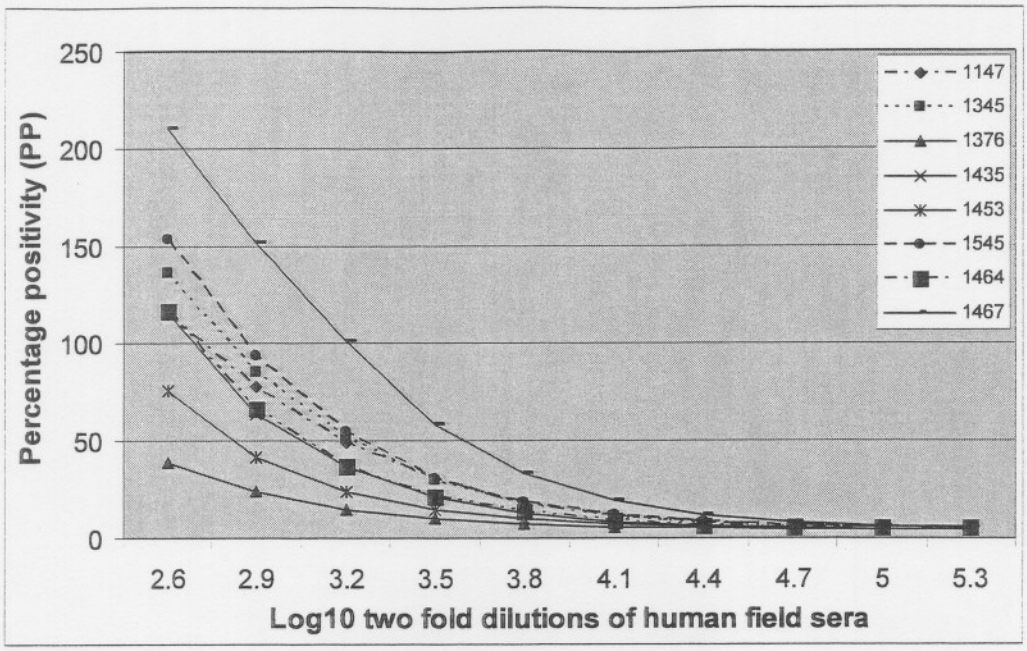


Figure 50. Titration of eight sera from RVF patients by I-ELISA using RVFV rN for detection of IgM antibodies. The symbol for each serum on the graph is displayed in the legend in the figure.

5.3.3 Evaluation of the recombinant RVFV nucleoprotein expressed in *E. coli* as antigen in an I-ELISA for detection of anti-RVFV antibodies in experimental sheep sera:

An experiment was done to compare the abilities of Protein G and rabbit anti-sheep IgG conjugates to monitor the immune responses of experimentally infected sheep in the indirect ELISA. The ELISA plate was coated overnight with a 1:2000 dilution of RVFV rN in carbonate/bicarbonate buffer. Control and test sera were added as indicated in Figure 39.

Sera from a sheep (sheep number 6154) that had previously been inoculated with RVFV under laboratory conditions were obtained from the SPU at the NICD. The immune response was monitored for 14 days and sera were collected daily from day 0 (pre-infection) to day 6 and again on days 8, 10 and 13. Duplicates of the sera was used in this ELISA experiment with IgG detected with protein G HRPO conjugate in one repeat and with rabbit anti-sheep IgG HRPO conjugate in the other. The PP value was calculated for each serum from each date of collection on both plates. The IgG antibody response of sheep 6154, as measured with conjugated protein G and rabbit anti-sheep IgG, can be seen in Figure 51. Figure 51 was drawn from the raw data from Table 18 and 19 in Appendix 1.

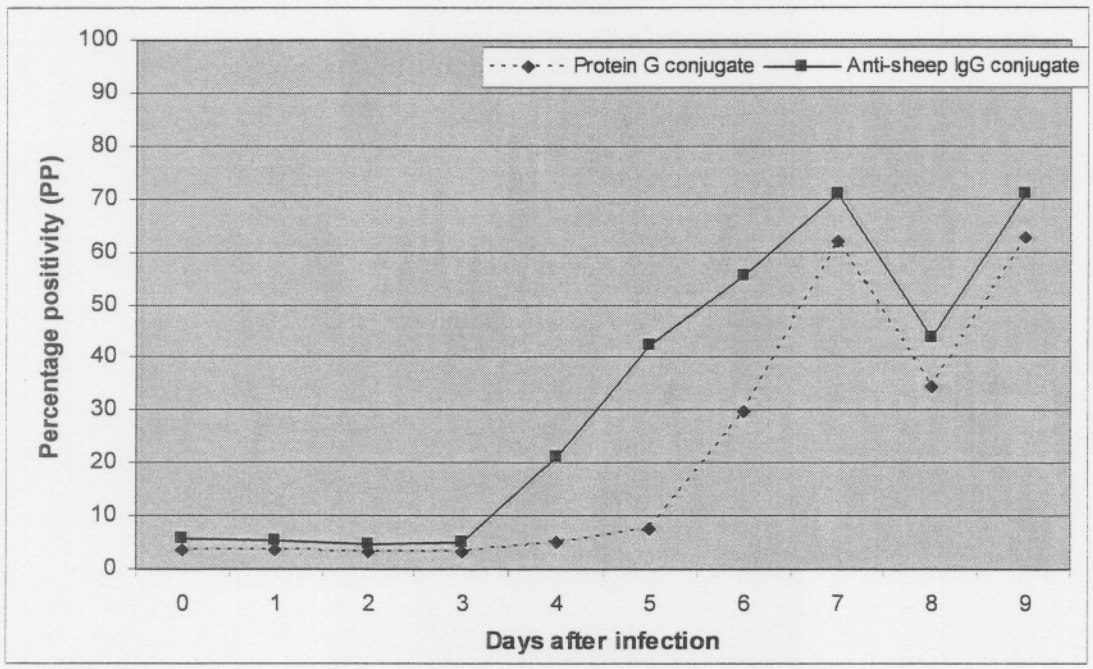


Figure 51. Monitoring if IgG responses in experimentally infected sheep by I-ELISA using two different HRPO-conjugates, Protein G and anti-sheep IgG. Immune response tracked by Protein G (▲) and the anti-sheep IgG (■).

From the graph it is clear that the sheep did not have any antibodies against RVFV before the experimental infection. The anti-sheep IgG curve shows that seroconversion occurred at day 3, as the PP value of the serum had increased sharply. The Protein G curve, however, only shows a comparable increase in PP value between day 4 and 5. This demonstrates that the specific anti-sheep IgG conjugate is more sensitive than Protein G in detecting low levels of IgG antibodies. Thus, it was decided to use the specific anti-species conjugates for further IgG testing.

An experiment was done to monitor the IgG antibody responses of 10 sheep vaccinated with the live-attenuated Smithburn vaccine strain of RVFV. Sera were collected daily from day 0 (pre-vaccination) to day 20 in 7 of the vaccinated sheep, and from day 0 to day 32 in the other three. The average PP value for all 10 sheep was determined for each collection day from the raw data given in Tables 20 - 26 in Appendix 1, and the daily averages are shown in Figure 52.

On average, the seroconversion occurred between day 6 and 8 (Figure 52). This can be seen by the increase in PP value at this collection date. The curve shows the increase in PP values over time after vaccination as the IgG antibody levels increase as expected.

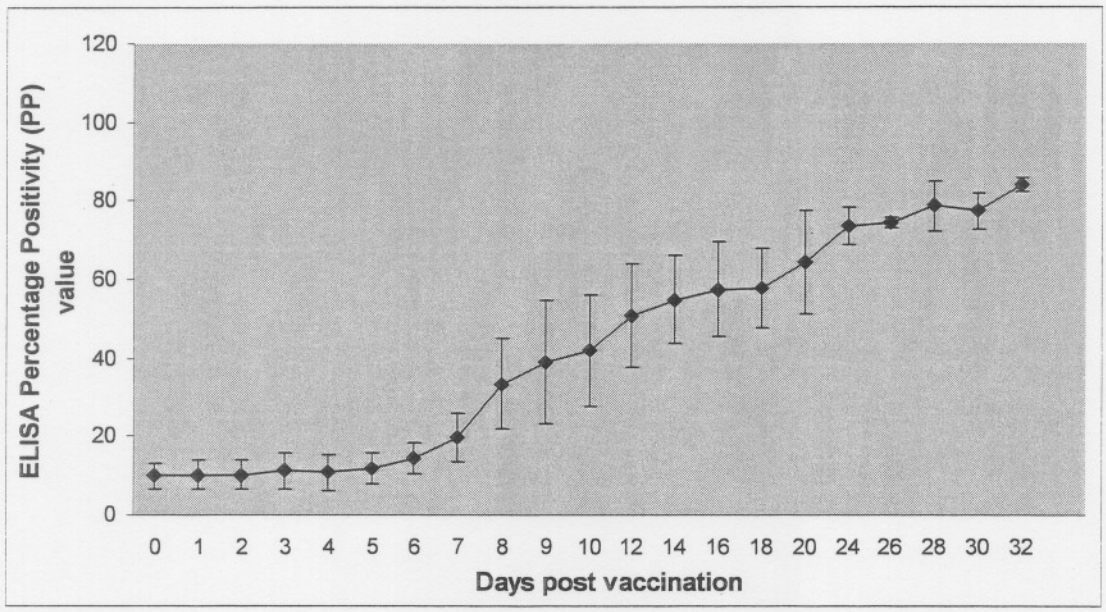


Figure 52. Mean +/- 1 S.D. of IgG antibody levels in 10 sheep vaccinated with the live-attenuated Smithburn vaccine strain.

The IgM antibody responses in the 10 vaccinated sheep were also analyzed using the indirect ELISA format with a 1:500 dilution of the RVFV rN antigen. The average PP value for all 10 sheep was determined for each collection day from the raw data given in Tables 30 - 36 (Appendix 1), and summarized in Figure 53.

The IgM levels in the sheep increased sharply from day 4-5 (Figure 53). The IgM antibodies in the sheep sera reached a peak on day 9 post infection, after which the antibodies decrease to a lower level which was not surprising considering the transient nature of this type of antibody after vaccination or natural infection.

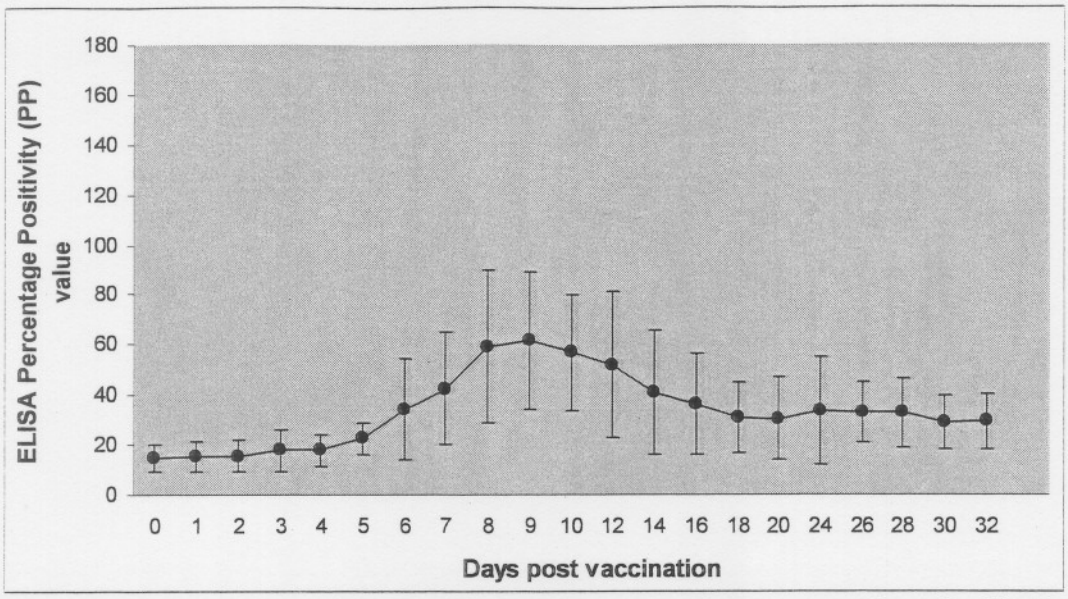


Figure 53. Mean +/- 1 S.D. of IgM antibody response in 10 sheep vaccinated with the live-attenuated Smithburn RVFV vaccine strain.

An experiment was done to monitor the immune responses in 8 sheep that were infected with a wild type strain of RVFV. Sera were collected immediately before the experimental inoculation (day 0) and on the following days: 1-14, 21, 28, 42, 51, 56, 63, 70 and 77 post infection. The average PP value for all 8 sheep was determined for each collection day from the raw data given in Tables 37 - 41 (Appendix 1), and summarized in Figure 54.

On average, seroconversion occurred between day 4 and 5. This is indicated by the increase in PP value on this collection date. The curve shows the increase in PP values over time after the vaccination as the IgG antibody levels increase. When comparing Figure 54 to Figure 52 one can see that the immune response is much higher in infected sheep than in vaccinated sheep, which clearly demonstrates differences between immunological response in sheep inoculated with attenuated virus and wild type virus. Seroconversion was detectable 2 days earlier in infected sheep than in vaccinated sheep.

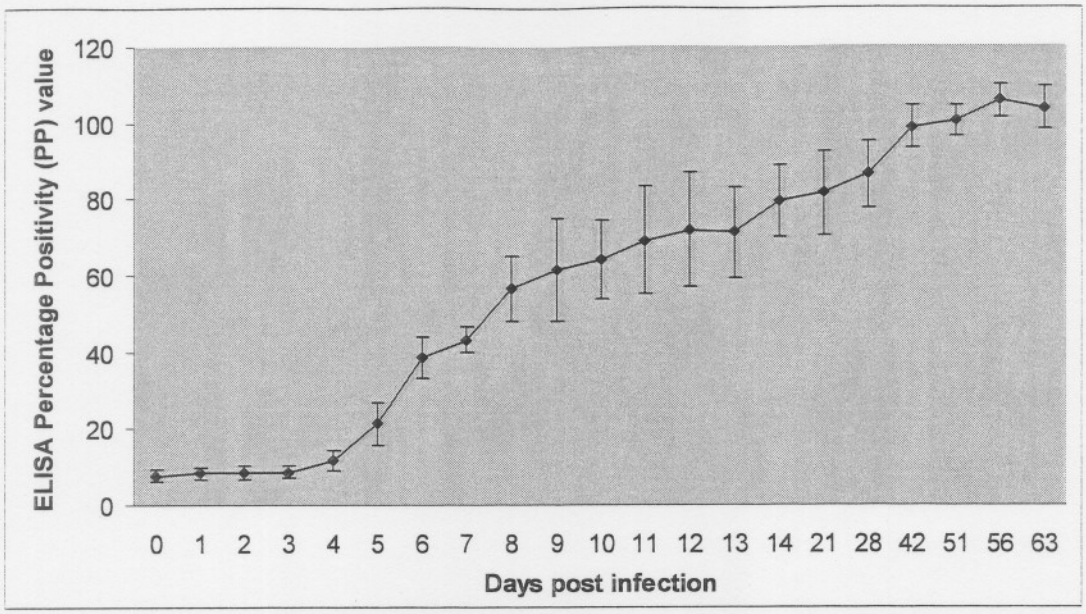


Figure 54. Mean +/- 1 S.D. of IgG antibody response in eight sheep experimentally infected with wild type RVFV strain.

The IgM antibody response in the 8 RVFV infected sheep was also analyzed using the indirect ELISA with a 1:500 dilution of the RVFV rN antigen. Sera were collected on the day of infection, and then on the following days post infection (d.p.i.): 1-14, 21, 28, 42, 51, 56, 63, 70 and 77 post infection. The daily average PP values were determined for all 8 sheep from the raw data given in Tables 42 - 46 (Appendix 1), and summarized in Figure 55.

The IgM response increased sharply from 4 d.p.i and reached a peak by day 7-10 d.p.i, followed by a gradual decreas to low levels. When comparing Figure 55 with Figure 53 one can see that the IgM antibody response in infected sheep is higher than in sheep vaccinated with the Smithburn vaccine strain.

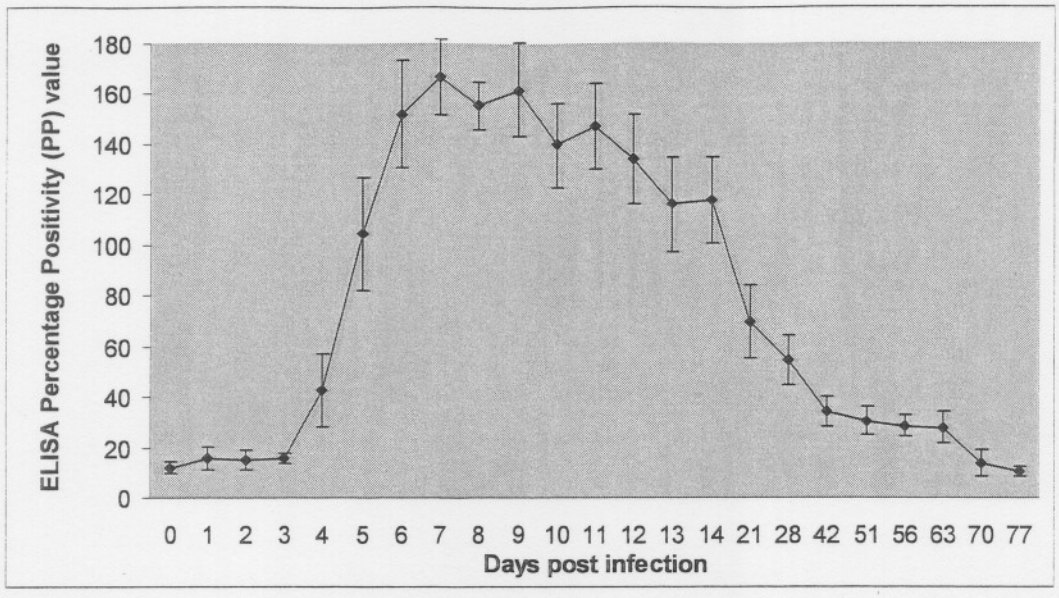


Figure 55. Mean +/- 1 S.D. of IgM antibody response in eight sheep experimentally infected with wild type RVFV strain.

5.4 Summary

There is an increasing international demand for the development of safe, cost-effective and robust serological techniques for the detection of RVFV infection in humans and animals. The applicability of various ELISAs for detection of antibodies in humans and animals against RVFV has been shown (Niklasson et al., 1984; Paweska et al., 2003b). However, these methods are based on the use of inactivated whole virus as an antigen. Production of such antigens is expensive and laborious and poses a health risk to laboratory personnel. Therefore, traditional antigen production is restricted to high biocontainment laboratories (BSL3 or BSL4). The feasability of the pET32(a)+ bacterially expressed RVFV nucleoprotein as an antigen was evaluated in an indirect ELISA (I-ELISA) format. The I-ELISA is difficult to validate because there is an inherent amplification of non-specific signal from contaminating components in the antigen preparation (Paweska et al., 2003b). Thus the antigen for the I-ELISA has to be of very high quality. The purified rN RVFV antigen contained no noticeable non-specific proteins (Figure 36). The two proteins present after purification represent the 42kDa fusion nucleoprotein which includes the tag added by pET32(a)+ and a very small amount of the second protein of 27kDa which most probably represents the nucleoprotein without the tag. As explained in Summary 4.4, this is possibly due to the formation of N dimers. Wild type recombinant N protein without the tag is also expressed because of the presence of an ATG start codon in the cloned insert of RVFV N gene into pET32(a)+.

During optimization of I-ELISAs based on pET32(a)+ expressed recombinant protein it was found that overnight coating at 4°C using carbonate/bicarbonate buffer pH9.6 yielded the best results (Figures 41 and 42). The effect of purification of the antigen on I-ELISA readings is shown in Figure 43. From this figure one can see that the positive-negative ratio is low at low dilutions of the antigen, then increases as the antigen becomes more diluted and reaches a peak, after which it then decreases again as the antigen is diluted further. These results represent an expected, characteristic slope for normally titrating biological reagents. Diluting test and control sera to a relatively high dilution (usually 1:400) minimizes any nonspecific signal which might arise due to potential cross-reactive antibodies against other phleboviruses present in the serum (J.T. Paweska, personal communication). The same principle applies to dilution of the antigen, which minimizes cross-reactivity with possible *E. coli* antigens present in the RVFV rN preparation.

The ability of the recombinant nucleoprotein antigen to monitor different concentrations of antibodies is shown in Figures 49 and 50. Figure 49 tracks the two-fold serial dilutions of 8 individual sera from RVF patients known to be positive for IgG antibodies with VN titres ranging from 1:10 to 1:1280. There was an expected decrease in the levels of IgG antibodies in all 8 sera with increasing dilution factor. There is also a clear distinction between sera with differing antibody concentrations (Figure 49). Figure 50 shows the results from the two-fold serial dilutions of 8 individual sera from 8 humans, naturally infected during the 1998-1999 RVF outbreak in East Africa, for IgM antibodies against RVFV. There was an expected decrease in the concentrations of IgM antibodies in all 8 sera with increasing dilution factor. There is also a clear distinction between sera with differing concentrations of IgM antibodies (figure 50). When comparing Figures 49 and 50 it can be seen that there is generally a higher concentration of IgM antibodies in the naturally infected human sera than there is IgG

antibodies in the vaccinated human sera. This most probably is due to the fact that natural infections stimulate higher humoral immunological responses than vaccination with non-replicating inactivated RVFV. IgM antibodies are usually the first to be produced during RVFV infection and also the first to decline within a few months after RVFV exposure (Woods et al., 2002).

The applicability of the RVFV recombinant antigen for detection of anti-RVFV antibodies in humans, sheep and cattle control sera was clearly demonstrated in this study (Figure 44). The antigen was shown to easily detect specific antibodies in human and sheep sera. There was, however, low ELISA signal and high ELISA background when testing a limited panel of cattle control sera. This could be attributed to the phenomenon of "sticky sera" (Niklasson et al., 1984b) which is caused by non-specific antibodies in the sera binding to the antigen. The applicability of a HRPO conjugated Protein G for I-ELISA was also tested. Protein G binds to any IgG antibody at their Fc site. Because of this ability it could theoretically be used for the detection of antibodies in different species (human, sheep, cattle etc.) in a single test format. Therefore, it could simplify serological diagnosis during a country-wide survey or outbreak of RVFV. Protein G was shown to work very well for sheep control sera, but was less effective for testing human and cattle sera (Figure 45). Possibly sheep IgG has a higher specificity for Protein G than human and cattle sera. Another possibility is that the sheep control sera could have less cross-reacting antibodies than the human and cattle sera. This assumption is supported by better ELISA performance with anti-sheep IgG conjugate than with anti-human IgG and anti-bovine IgG conjugates (Figure 44). It also correlates with the raw OD values (Table 17) where the sheep sera have much lower readings for the negative control (C-) sera than human and cattle sera.

A comparison of the ability of Protein G and rabbit anti-sheep IgG to track the immune response of an experimentally infected sheep was tested (Figure 51 and Tables 18 and 19). The ELISA signal with rabbit anti-sheep IgG was higher than that of protein G. The ELISA signal using rabbit anti-sheep IgG also detects IgG antibody development earlier (day 3-4) than with Protein G (day 4-5) which means earlier detection of seroconversion. It was therefore decided to use anti-species specific conjugated antibodies for further evaluation of ELISAs.

The I-ELISA was used to study the kinetics of IgG and IgM responses in vaccinated and experimentally infected sheep (Figure 52 and 53). IgG seroconversion in vaccinated sheep was detectable at day 6-7 after vaccination, and thereafter IgG antibodies steadily increased. The IgM test showed an increase and subsequent decrease in IgM antibodies in the sheep. This was expected because anti RVFV IgM antibodies generally appear around day 5 and decrease by 50% within 6 weeks (Woods et. al., 2002). In contrast, IgG antibodies remain indefinitely (Barnard, 1979). When comparing these results to previous results obtained from the same sheep specimens using IgG sandwich and IgM capture ELISAs based on traditional whole virus antigen (Paweska *et al.*, 2003a), the same results and graph characterisics were obtained. Seroconversion of the sheep was detectable at the same day post vaccination by the recombinant ELISA and traditional ELISAs.

The IgG and IgM antibody responses in 8 experimentally RVFV infected sheep were demonstrated using the recombinant N-based I-ELISA (Figures 54 and 55). The test showed IgG production at day 4-5, followed by an increase in IgG antibody levels for all the sheep tested. The IgM I-ELISA showed

an increase and subsequent decrease in IgM antibodies in the sheep sera. IgM antibodies appeared and increased dramatically from day 3 and thereafter decreased to pre-infection levels by day 70 post-infection. This was expected because anti RVFV IgM antibodies generally appear around day 5 and decrease by 50% within 6 weeks (Woods et. al., 2002). When comparing these results to previous results obtained from the same sheep specimens using IgG sandwich and IgM capture ELISAs based on traditional whole virus antigen (Paweska *et al.*, 2003a), the same results and graph characterisics were obtained. Seroconversion of the sheep was detectable at the same day post vaccination by the recombinant ELISA and traditional ELISAs. It is important to note that the rN-based I-ELISA was capable of detecting differences in antibody kinetics between vaccinated and infected sheep [Figures 52 and 53 (vaccinated sheep); Figures 54 and 55 (infected sheep)]. The level of IgG and IgM antibodies were higher in infected sheep than in vaccinated sheep. This could be attributed to the fact that the Smithburn strain is an attenuated vaccine strain of the virus and therefore, most likely it is much less immunogenic than the wild type virus. These same differences in antibody levels between vaccinated and infected animals were also detectable with traditional antigen based IgG sandwich and IgM capture ELISAs (Paweska *et al.*, 2003a).

The applicability of the RVFV recombinant nucleoprotein was also shown for other ELISA formats. Preliminary experiments were done to evaluate the IgG sandwich ELISA (Figure 47) and IgM capture ELISA for detecting antibodies in sheep sera (Figure 48). Although the results are limited, they are promising and indicate the potential use of the recombinant N antigen in these ELISAs as well. Both ELISAs yielded the expected pattern of results. Compared to IgG sandwich and IgM capture ELISAs based on traditional sucrose acetone extracted infected mouse brain antigens, the limited readings of strong positive serum was lower when using the rN protein as antigen (data not shown), but the methods cannot be compared directly because the rN protein was not tested in the dilutions normally used for the traditional antigen.

CHAPTER 6

CONCLUDING REMARKS

In this chapter the main results of this study are summarized, as well as some envisaged future developments discussed. The findings of this study have been submitted for publication in the Journal of Virological Methods (see title and abstract in Appendix 3) and have been presented at the Molecular and Cell Biology Group symposium hosted by the University of Witwatersrand Medical School, held on the 5th of October 2006 at the Johannesburg Hospital Auditorium (see title and abstract in Appendix 4). Further work resulting from this study will also be presented in poster form at the NICD academic day, held on the 28th of November 2006, by Dr. J.T. Paweska (see Appendix 5).

Rift Valley fever virus (RVF) is an emerging viral disease that poses a significant threat to humans and livestock. Historically it is endemic to sub-Saharan Africa but has recently spread outside this region which is of great international veterinary and medical concern. The most important examples of RVF emerging outside its traditional boundaries are outbreaks in Egypt in 1977 and 1993, and most recently the first outbreaks outside Africa, in the Arabian Peninsula in 2000 and 2001 (Jupp et al., 2002; Shoemaker et al., 2002). The causative agent, Rift Valley fever virus (RVFV), was first isolated in Kenya in 1931 (Daubney and Hudson, 1931; Battles and Dalrymple, 1988). The virus is transmitted mainly by bites from infected mosquitoes and, therefore, extensive outbreaks occur mainly during high rainfall periods when competent mosquito vectors are abundant. Humans can also be infected by exposure to tissues and blood from infected animals. Infections in livestock are characterized by acute hepatitis, high abortion rates and high mortality rates among young animals. Human symptoms can range from mild febrile illness to more severe clinical presentations including retinal degeneration, encephalitis, fatal hepatitis and hemorrhagic fever (Swanepoel and Coetzer, 2004).

Diagnosis of RVFV infection can be achieved by virus isolation, nucleic acid techniques and detection of viral antigens and antibodies (Gerdes, 2004). These methods are either highly sophisticated or extremely laborious. Traditional serological tests include haemaggiutination-inhibition, complement fixation, indirect immunofluorescence and virus neutralization (Paweska et al., 2005b; Shope and Sather, 1979; Swanepoel et al., 1986). These methods pose a potential health risk to laboratory personnel, thus their use is restricted to high biocontainment laboratories outside endemic regions. The tests are unable to distinguish between different classes of immunoglobulins (Paweska et al., 2005b). Various ELISA methods have been developed and validated for diagnosis of RVFV (Meegan et al., 1987; Niklasson et al., 1984; Paweska et al., 2003 a & b; Paweska et. al., 2005 a & b) but they are based on the use of inactivated virus as antigen and, therefore, pose a poteintial biohazard risk.

RVFV is classified in the genus *Phlebovirus* of the family *Bunyaviridae*. The genome consists of negative sense RNA comprised of three segments; L, M and S. The S segment follows an ambisense coding strategy to encode for the nucleoprotein (N) and a non-structural protein (NSs) (Ihara, Akashi and Bishop, 1984; Giorgi et al., 1991).

The aim of this project was to clone and express the RVFV nucleoprotein and to evaluate its potential as a diagnostic antigen in an ELISA. The main advantage of using a recombinant antigen in an ELISA would be the elimination of health risks to diagnosticians which would facilitate the use of ELISAs outside endemic regions.

Viral RNA was extracted from cell culture supernatant containing the Zim688/78 strain of RVFV, which was isolated from an infected bovine in Zimbabwe during the 1978 outbreak. The gene encoding the nucleoprotein was amplified by RT-PCR using specifically designed primers. The purified N gene amplicon was cloned into the vector, pGEM-T Easy (Promega), using the A/T cloning strategy. The recombinant plasmid containing the RVFV N gene and the RT-PCR amplicon were sequenced to determine the nucleotide sequence of the Zim688/78 N gene. A region of the M segment encoding a part of the glycoprotein G2 of RVFV Zim688/78 was amplified as a positive control with RT-PCR using primers obtained from the Special Pathogens Unit of the National Institute for Communicable Diseases. This amplicon was also sequenced. In this study parts of the M and S genome segments of the Zim688/78 strain encoding the N and G2 proteins respectively were shown to be closely related to different RVFV strains. The N gene sequence of Zim688/78 was compared to the 7 other published sequences and found to be most closely related to the N gene sequence of the Clone 13 strain of RVFV. Zim688/78 was also found to have 5 unique changes in its nucleic acid sequence compared to the 7 published strains. These nucleic acid changes are silent mutations and do not affect the amino acid sequence. The amino acid sequence of the RVFV Zim688/78 strain is identical to that of the MP12 strain. The partial G2 sequence of the Zim688/78 strain was also compared to corresponding regions in the sequences of other published strains, and found to be closest related to the BEgy93, HEgy93 and ZH548 strains.

For baculovirus expression of the RVFV N protein, the N gene was cloned into baculovirus vectors utilizing the restriction enzyme sites, BamHI and XhoI, that were engineered into the RT-PCR primers, and matching sites in pFastBac1, pFastBacHT-B and pBACgus-1. pFastBac1 and pFastBacHT-B can be used in the Bac-to-Bac® Baculovirus expression system (Luckow and Summers, 1989), which is based on the transposition of an expression cassette generated in bacteria into a specific site on a baculovirus bacmid. pBACgus-1 can be used in the BacMagic DNA system (Kitts, Ayres and Possee, 1990), which utilizes a linearized AcMNPV genome, with a portion of an essential open reading frame deleted and a bacterial artificial chromosome inserted in place of the polyhedrin coding region. Baculovirus expression systems have been used successfully in the past to express RVFV recombinant antigens. Schmaljohn *et. al.* (1989) used baculovirus expression for the production of recombinant proteins (G1 and G2) from the M genome segment of RVFV. These recombinants were used to study their application as immunogens for vaccination and antigens for serological tests (Schmaljohn *et. al.,* 1989).

The RVFV recombinant baculovirus vector constructs were checked by restriction enzyme analysis and sequencing. Confirmed recombinant plasmids were used to prepare recombinant baculoviruses in SF9 insect cells. These recombinant viruses were used to infect SF9 cells and evaluate expression of the nucleoprotein in these cells. The level of expression from pFastBacHT-B was relatively low and the largest part thereof was insoluble. The level of expression from pFastBac1 was higher than from

pFastBacHT-B and it was also slightly more soluble. Recombinant bacmid DNA from both expression vectors was sent to the commercial company, Biovac (Pinelands, South Africa), for further optimization and up-scaling of expression. Results are not yet available. Expression with the BacMagic System was not tested. Due to the high cost and labor intensiveness of the baculovirus system, it was decided to also evaluate expression of rN RVFV in bacterial expression systems.

For bacterial expression of the RVFV N protein, the N gene was cloned into bacterial vectors utilizing the restriction enzyme sites, BamHI and XhoI, that were engineered into the RT-PCR primers, and matching sites in pET32(a)+ and pGEX4T-1. Bacterial expression has been utilized in the past to express RVFV antigens. Keegan and Collet (1986) expressed portions of the G2 glycoprotein of RVFV in order to determine the amino acid sequences of antigenic determinants on the G2 protein. Production of recombinant nucleoprotein has been accomplished previously, but these proteins were not used as antigens in I-ELISA. In one case the recombinant N protein was produced by using Semliki Forest virus as a vector for expression in mosquito cells. This protein was used to examine the effect thereof on the replication of the virus in mosquito cells (Billecocq et al., 2000). It was also used for the identification of monoclonal antibodies against RVFV N protein (Zaki et al., 2005). The vector pET32(a)+ makes use of the T7 expression system, thus the target gene is under control of the T7 promoter, which is IPTG inducible. The vector pGEX4T-1 utilizes the Glutathione S-transferase (GST) gene fusion system, and the target gene is under control of the *tac* promoter, which is also IPTG inducible.

The recombinant bacterial expression vector constructs were checked by restriction enzyme analysis and sequencing. The confirmed pET32(a)+ recombinant (pRVFN-ET32) was used for expression of the N protein in two different cell lines. Expression of N with pET32(a)+ in NaCl inducible BL21+S was very low. Lower expression levels are expected with NaCl induction (Bhandari and Gowrishankar, 1997). However, expression of N with pET32(a)+ in IPTG inducible Origami(DE3) cells was higher and the product was also completely soluble. The recombinant nucleoprotein (fused with the tags added by pET32a+) was also easily purified by affinity purification using the 6 x Histidine tag. Two proteins, a 42 kDa and a 27 kDa protein, were present after purification (instead of the expected one of 42 kDa). The 27 kDa protein is most likely the nucleoprotein without the fusion tag. The N terminal primer was designed to incorporate the N gene's own ATG codon, thus translation could occur from this codon. According to the literature (Le May et al., 2005) the N protein is able to form dimers with itself, which would explain why the N protein without tag was co-purified with the tagged fusion N protein. Expression of N protein with pGEX4T-1 in IPTG inducible cells BL21 was also efficient. However, the product was insoluble. One could optimize the growth rate of the cells during induction by lowering the temperature and lowering aeration to increase solubility of the product. However, it was decided to use the pET32(a)+ system for further expression of recombinant N protein because of its solubility and ease of purification.

The applicability of the pET32(a)+ recombinantly expressed RVFV N protein (rN) as an antigen in various ELISA forms for detection of antibodies in humans and animals was evaluated. Different coating procedures and buffers were tested. Overnight coating of MaxiSorp immunoplates at 4°C using carbonate/bicarbonate buffer pH 9.6 gave the best results in the I-ELISA. The importance of

purification of the antigen for I-ELISA was clearly demonstrated. The fact that RVFV rN protein can be directly coated on an ELISA plate presents a significant advantage to the traditional antigen. It has been reported that whole inactivated virus does not easily coat onto ELISA plates (Paweska et. al., 2005a). The applicability of the I-ELISA with the rN protein as antigen to measure IgG and IgM antibodies in serial dilutions of known positive sera was shown. The test revealed the expected decreasing curve pattern and was able to distinguish between sera containing high, low and no levels of antibodies against RVFV. The ELISA formats currently in use for the diagnosis of RVFV in humans are a sandwich ELISA for IgG and a capture ELISA for IgM (Paweska, Burt and Swanepoel, 2005). Both these methods are more laborious than the I-ELISA and are based on the use of inactivated whole virus as an antigen. The I-ELISA based on the rN protein developed in this study is a significant improvement on both these methods because results are obtained much quicker and with much less labour. Moreover, production of recombinant antigen is easier, cheaper, quicker and safer compared to the traditional production of inactivated whole virus antigen. The antigen is also safe to use by laboratory personnel that are not immune to RVFV and allows its application in BSL-2 laboratories outside RVFV endemic regions when inactivated sera are tested.

The ability of the I-ELISA using the rN to detect antibodies to RVFV in animals was also explored. The presence of antibodies against RVFV in sheep control sera was readily detectable, and strong positive control sera were easily distinguishable from low positive and negative sera. The detection of antibodies in bovine sera was not as efficient as for sheep and human sera. This could probably be attributed to the "sticky sera" phenomenon found in African livestock (Niklasson et al., 1984b). This phenomenon is caused by non-specific antibodies in the sera binding to the antigen, which is then also detected by the HRPO conjugated antibody. The possibility of using Protein G as a single HRPO conjugated detection system was also explored but the results obtained indicate that specific anti-species HRPO conjugated antibodies were more effective in detecting lower levels of antibodies. The ability of the rN-based I-ELISA to track IgG and IgM immune responses in sheep sera was investigated. The IgG and IgM antibodies at various days post-vaccination of ten sheep vaccinated with the Smithburn vaccine strain of RVFV were monitored. It was found that the rN based I-ELISA was able to measure and show the expected increase in IgG antibodies in sheep sera after vaccination. The test was also able to show the expected kinetics of IgM immune response, with an increase in IgM antibodies from day 4-5 post-vaccination and gradual decrease to pre-vaccination levels at 43 days post-vaccination. These results compared well with results from ELISAs based on traditional antigen done on the same specimens (Paweska *et al.*, 2003a). The same analysis was done on the sera from 8 sheep experimentally infected with wild type strain of RVFV. The same expected increase in IgG and IgM antibodies post-infection was seen as for the vaccinated sheep, and also the subsequent decrease in IgM antibodies. However, the level of antibodies in the infected sheep sera was much higher than in the vaccinated sheep sera. This could most probably be attributed to the fact that the Smithburn strain of RVFV is an attenuated vaccine strain which is less immunogenic compared to pathogenic strains. These results indicated that the rN-based I-ELISA for detection of antibodies in sheep sera is superior to previously developed tests (IgG sandwich and IgM capture - Paweska et al., 2003a) in terms of the criteria mentioned previously for the human ELISA.

The production of the RVFV rN is technically easy, safe and highly repeatable. Contrary to antigens produced by traditional methods, the rN protein was found to be an excellent coating protein for the I-ELISA. The I-ELISA is superior to sandwich and capture ELISAs in the sense that the test takes less time to complete, uses fewer reagents and has fewer steps where mistakes could be made by the laboratory personnel conducting the test. The production of the rN protein of RVFV was shown to be very efficient and the protein a good antigen in I-ELISA for detection of antibodies in both humans and animals. Therefore, I believe that the aim of this project was achieved.

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APPENDIX 1

ELISA raw data

Table 12. Raw data of rN RVFV antigen titrations done on three different ELISA plates with 1 hour coating at 37°C

		1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600	1:51200	1: 102400
		1.7	2	2.3	2.6	2.9	3.2	3.5	3.8	4.1	4.4	4.7	5
Costar	Hu C++	2.81	2.671	2.229	1.361	0.778	0.462	0.281	0.19	0.144	0.132	0.086	0.085
	Hu C-	0.235	0.176	0.136	0.126	0.115	0.116	0.116	0.117	0.116	0.128	0.084	0.091
PolySorp	Hu C++	2.129	2.072	1.786	0.836	0.437	0.224	0.131	0.098	0.081	0.071	0.069	0.069
	Hu C-	0.156	0.127	0.115	0.106	0.109	0.105	0.114	0.109	0.102	0.099	0.096	0.099
MaxiSorp	Hu C++	2.952	2.808	2.349	1.359	1.082	0.699	0.312	0.271	0.184	0.142	0.12	0.13
	Hu C-	0.221	0.154	0.113	0.101	0.093	0.096	0.088	0.088	0.098	0.09	0.098	0.103

Table 13. Raw data of rN RVFV antigen titrations done on three different ELISA plates with overnight coating at 4°C

		1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600	1:51200	1: 102400
		1.7	2	2.3	2.6	2.9	3.2	3.5	3.8	4.1	4.4	4.7	5
Costar	Hu C++	2.145	2.311	2.11	1.903	1.374	0.907	0.513	0.331	0.24	0.181	0.15	0.151
	Hu C-	0.178	0.163	0.133	0.12	0.111	0.114	0.114	0.116	0.12	0.116	0.109	0.128
PolySorp	Hu C++	1.946	2.21	2.16	1.827	1.323	0.73	0.5	0.257	0.163	0.122	0.103	0.102
	Hu C-	0.173	0.166	0.14	0.137	0.138	0.124	0.127	0.124	0.126	0.129	0.133	0.143
MaxiSorp	Hu C++	2.454	2.367	2.255	2.08	1.505	1.109	0.757	0.513	0.333	0.24	0.183	0.158
	Hu C-	0.202	0.15	0.123	0.112	0.095	0.089	0.092	0.087	0.089	0.123	0.1	0.111

Table 14. Raw data of rN RVFV antigen titrations done on Maxisorp ELISA plate with overnight coating at 4°C comparing two different coating buffers

		1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600	1:51200	1: 102400
		1.7	2	2.3	2.6	2.9	3.2	3.5	3.8	4.1	4.4	4.7	5
PBS	Hu C++	2.631	2.608	2.53	2.129	1.455	0.973	0.583	0.37	0.307	0.165	0.141	0.142
	Hu C-	0.222	0.174	0.137	0.12	0.111	0.104	0.105	0.105	0.106	0.106	0.107	0.112
Carb/Bicarb	Hu C++	2.677	2.592	2.453	2.09	1.705	1.342	0.915	0.565	0.327	0.205	0.136	0.121
	Hu C-	0.186	0.146	0.107	0.091	0.088	0.084	0.084	0.083	0.084	0.09	0.089	0.103

Table 15. Raw data of rN RVFV antigen titrations done on Maxisorp ELISA plate with overnight coating in Carbonate/Bicarbonate buffer at 4°C comparing purified and unpurified antigens

	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600	1:51200	1: 102400	
	1.7	2	2.3	2.6	2.9	3.2	3.5	3.8	4.1	4.4	4.7	5	
Hu C++	2.783	2.731	2.791	2.789	2.669	2.629	2.518	2.174	1.653	1.065	0.661	0.4	Unpurified N
Hu C-	0.435	0.444	0.464	0.461	0.425	0.364	0.301	0.232	0.169	0.131	0.115	0.117	
Hu C++	2.677	2.592	2.453	2.09	1.705	1.342	0.915	0.565	0.327	0.205	0.136	0.121	Purified N
Hu C-	0.186	0.146	0.107	0.091	0.088	0.084	0.084	0.083	0.084	0.09	0.089	0.103	
Hu C++	1.506	1.491	1.583	1.616	1.281	0.873	0.639	0.386	0.306	0.201	0.205	0.226	E. Coli proteins
Hu C-	0.496	0.491	0.494	0.429	0.35	0.256	0.196	0.141	0.118	0.108	0.103	0.112	
Hu C++	0.085	0.078	0.078	0.077	0.075	0.076	0.077	0.076	0.076	0.077	0.077	0.087	Mock
Hu C-	0.097	0.095	0.093	0.093	0.092	0.097	0.094	0.095	0.093	0.091	0.101	0.099	

Table 16. Raw data of HRP conjugated antibody titrations done on Maxisorp ELISA plate with overnight coating in Carbonate/Bicarbonate buffer at 4°C comparing detection of antibodies in humans, sheep and cattle using rN RVFV antigen

Anti-species IgG HRPO	1:2000	1:4000	1:8000	1:16000	1:2000	1:4000	1:8000	1:16000	1:1000	1:2000	1:4000	1:8000
C++	2.707	2.309	1.72	1.112	1.617	1.133	0.685	0.389	1.491	1.041	0.656	0.412
C++	2.645	2.292	1.607	1.025	1.382	1.042	0.559	0.364	1.229	0.98	0.639	0.397
C+	1.455	0.969	0.599	0.341	0.454	0.297	0.166	0.134	0.63	0.391	0.247	0.165
C+	1.339	0.984	0.591	0.334	0.462	0.297	0.189	0.128	0.593	0.383	0.245	0.167
C-	0.367	0.23	0.141	0.1	0.12	0.095	0.077	0.066	0.26	0.178	0.129	0.106
C-	0.366	0.224	0.144	0.098	0.125	0.097	0.08	0.068	0.274	0.182	0.132	0.103
Mock	0.132	0.096	0.076	0.066	0.07	0.061	0.062	0.062	0.121	0.089	0.072	0.07
Mock	0.131	0.099	0.087	0.076	0.083	0.075	0.066	0.06	0.141	0.101	0.084	0.071
		Human sera				**Sheep sera**				**Bovine sera**		

Table 17. Raw data of HRP conjugated Protein G titrations done on Maxisorp ELISA plate with overnight coating in Carbonate/Bicarbonate buffer at 4°C, comparing detection of IgG antibodies in humans, sheep and cattle sera using rN RVFV as antigen

Protein G dilution	1:1000	1:2000	1:4000	1:8000	1:1000	1:2000	1:4000	1:8000	1:1000	1:2000	1:4000	1:8000
C++	1.221	1.16	0.929	1.012	1.765	1.993	1.4	1.261	1.258	1.358	1.193	1.056
C++	1.263	1.149	0.885	0.796	1.993	1.703	1.689	1.567	1.28	1.337	1.148	1.011
C+	0.464	0.408	0.463	0.292	0.521	0.44	0.311	0.274	0.614	0.442	0.428	0.381
C+	0.461	0.41	0.32	0.283	0.426	0.365	0.368	0.274	0.519	0.514	0.443	0.38
C-	0.208	0.151	0.137	0.121	0.164	0.131	0.106	0.094	0.231	0.182	0.156	0.145
C-	0.22	0.158	0.133	0.121	0.173	0.136	0.107	0.095	0.218	0.174	0.156	0.142
Mock	0.104	0.082	0.076	0.07	0.125	0.095	0.077	0.07	0.107	0.085	0.077	0.074
Mock	0.125	0.097	0.083	0.075	0.134	0.103	0.083	0.074	0.124	0.089	0.078	0.073
		Human sera				**Sheep sera**				**Bovine sera**		

Table 18 and 19. Raw data of IgG immune response of sheep 6154 done on Maxisorp ELISA plate with overnight coating of RVFV rN antigen in Carbonate/Bicarbonate buffer at 4°C, comparing protein G and anti-sheep IgG conjugates

Protein G

	Control sheep sera		26.08.98	27.08.98	28.08.98	29.08.98	30.08.98	31.08.98	01.09.98	03.09.98	05.09.98	08.09.98	**Date collected**
C++	2.407	2.339	0.084	0.086	0.076	0.075	0.12	0.186	0.721	1.493	0.837	1.51	Sheep 6154 1998
C++	2.397	2.331	0.086	0.079	0.075	0.072	0.113	0.177	0.686	1.447	0.79	1.468	
C+	0.478	0.475	0.083	0.078	0.079	0.066	0.07	0.07	0.156	1.485	0.827	0.766	
C+	0.465	0.476	0.085	0.084	0.079	0.067	0.073	0.071	0.162	1.508	0.801	0.75	Sheep 6572 1998
C-	0.097	0.093	0.079	0.091	0.079	0.083	0.098	0.128	0.151	0.214	0.393	0.516	
C-	0.093	0.098	0.08	0.091	0.078	0.083	0.101	0.123	0.148	0.204	0.383	0.519	Sheep 1 2006
Mock	0.063	0.062	0.359	0.1	0.11	0.116	0.12	0.116	0.137	0.348	1.907	2.196	
Mock	0.065	0.064	0.404	0.109	0.118	0.124	0.129	0.124	0.145	0.357	2.032	2.246	Sheep 3 2006
			03.03.06	04.03.06	05.03.06	06.03.06	07.03.06	08.03.06	09.03.06	10.03.06	13.03.06	16.03.06	**Date collected**

Rabbit anti-sheep IgG

	Control sheep sera		26.08.98	27.08.98	28.08.98	29.08.98	30.08.98	31.08.98	01.09.98	03.09.98	05.09.98	08.09.98	**Date collected**
C++	1.765	1.753	0.1	0.091	0.084	0.087	0.368	0.746	0.96	1.235	0.783	1.086	Sheep 6154 1998
C++	1.836	1.716	0.097	0.092	0.084	0.09	0.382	0.74	0.992	1.26	0.759	1.413	
C+	0.511	0.518	0.1	0.095	0.13	0.076	0.136	0.108	0.476	1.282	0.818	0.962	
C+	0.609	0.517	0.101	0.097	0.093	0.077	0.137	0.104	0.474	1.376	0.853	0.813	Sheep 6572 1998
C-	0.105	0.099	0.099	0.131	0.105	0.125	0.169	0.211	0.231	0.304	0.502	0.56	
C-	0.103	0.097	0.101	0.128	0.108	0.125	0.167	0.201	0.233	0.304	0.477	0.538	Sheep 1 2006
Mock	0.062	0.064	0.729	0.204	0.243	0.25	0.287	0.366	0.517	0.915	1.63	1.727	
Mock	0.062	0.061	0.751	0.219	0.25	0.264	0.306	0.353	0.569	0.977	1.669	1.743	Sheep 3 2006
			03.03.06	04.03.06	05.03.06	06.03.06	07.03.06	08.03.06	09.03.06	10.03.06	13.03.06	16.03.06	**Date collected**

Table 20. Raw data of IgG immune response of 10 Smithburn vaccinated sheep using a Maxisorp ELISA plate with overnight coating of RVFV rN as antigen in Carbonate/Bicarbonate buffer at 4°C

C++	1.384	0.972	0.209	0.214	0.241	0.269	0.317	0.294	0.318	0.407	0.674	1.075	
C++	1.4	1.242	0.155	0.202	0.167	0.208	0.235	0.225	0.182	0.268	0.48	0.78	**Sheep**
C+	0.446	0.466	0.731	0.75	0.855	1.011	0.926	1.061	0.972	1.004	0.983	1.127	**3290**
C-	0.097	0.095	0.645	0.676	0.804	0.862	0.873	1.001	0.919	0.92	0.942	1.056	
C	0.095	0.087	0.899	0.116	0.109	0.121	0.111	0.106	0.168	0.173	0.252	0.675	
Mock	0.062	0.056	0.859	0.107	0.103	0.112	0.11	0.104	0.15	0.173	0.266	0.606	**Sheep**
	0.549	0.691	0.677	0.707	0.766	0.722	0.771	0.791	0.8	0.798	0.809	0.979	**3291**
	0.626	0.65	0.691	0.732	0.837	0.761	0.741	0.801	0.808	0.934	0.841	1.004	

Table 21.

C++	1.438	1.43	0.106	0.131	0.108	0.119	0.121	0.114	0.155	0.303	0.604	0.613	
C++	1.459	1.354	0.096	0.103	0.1	0.107	0.101	0.106	0.139	0.24	0.543	0.578	**Sheep**
C+	0.489	0.485	0.586	0.578	0.808	0.656	0.691	0.713	0.73	0.773	0.733	0.827	**3292**
C+	0.462	0.467	0.552	0.627	0.771	0.661	0.695	0.702	0.709	0.741	0.697	0.766	
C-	0.105	0.092	0.099	0.094	0.1	0.109	0.119	0.157	0.232	0.409	0.575	0.783	
C-	0.103	0.09	0.092	0.093	0.099	0.104	0.116	0.158	0.233	0.384	0.496	0.719	**Sheep**
Mock	0.062	0.06	1	0.691	0.676	0.74	1.069	0.726	0.767	0.778	0.795	0.873	**3293**
Mock	0.061	0.061	0.795	0.657	0.708	0.794	0.729	0.786	0.767	0.829	0.866	0.906	

Table 22.

C++	1.467	1.423	0.177	0.151	0.163	0.153	0.189	0.194	0.397	0.635	0.793	0.849	
C++	1.415	1.452	0.135	0.11	0.142	0.136	0.162	0.179	0.37	0.609	0.754	0.988	**Sheep**
C+	0.523	0.497	0.844	0.825	0.896	0.885	0.92	0.936	0.947	0.907	0.944	1.02	**3297**
C+	0.514	0.426	0.77	0.816	0.888	0.88	0.931	0.92	0.925	0.863	0.935	1.041	
C-	0.115	0.102	0.057	0.055	0.055	0.056	0.055	0.055	0.056	0.056	0.055	0.059	
C-	0.097	0.092	0.056	0.055	0.056	0.056	0.056	0.057	0.056	0.056	0.056	0.059	**Mock**
Mock	0.059	0.066	0.056	0.056	0.055	0.063	0.058	0.056	0.055	0.057	0.055	0.058	
Mock	0.06	0.058	0.06	0.058	0.058	0.059	0.06	0.059	0.059	0.058	0.058	0.061	

Table 23.

C++	1.397	1.37	0.115	0.146	0.131	0.138	0.131	0.142	0.206	0.189	0.262	0.369	
C++	1.453	1.306	0.101	0.131	0.119	0.131	0.124	0.124	0.186	0.168	0.238	0.385	**Sheep**
C+	0.53	0.458	0.264	0.422	0.436	0.506	0.507	0.513	0.502	0.531	0.574	0.577	**3296**
C-	0.115	0.108	0.249	0.411	0.42	0.493	0.514	0.509	0.505	0.531	0.558	0.606	
C-	0.101	0.096	0.555	0.101	0.1	0.101	0.087	0.111	0.14	0.186	0.226	0.373	
Mock	0.06	0.056	0.485	0.1	0.096	0.093	0.084	0.107	0.126	0.183	0.216	0.341	**Sheep**
	0.403	0.429	0.432	0.474	0.557	0.536	0.605	0.586	0.6	0.714	0.609	0.866	**3299**
	0.402	0.432	0.439	0.476	0.548	0.56	0.591	0.679	0.768	0.628	0.713	0.725	

Table 24.

C++	1.228	1.25	0.21	0.212	0.22	0.221	0.211	0.203	0.206	0.215	0.244	0.259	
C++	1.225	1.256	0.199	0.19	0.203	0.208	0.189	0.201	0.194	0.188	0.222	0.225	
C+	0.434	0.42	0.279	0.618	0.719	0.758	0.736	0.73	0.724	0.786	0.835	0.83	**Sheep**
C+	0.407	0.425	0.26	0.56	0.681	0.705	0.689	0.71	0.713	0.759	0.794	0.858	**3294**
C-	0.104	0.099	0.926	0.933	0.939	0.973	0.966	0.948	1.01	0.986	0.96	0.944	
C-	0.098	0.095	0.869	0.877	0.93	0.954	0.967	0.937	0.97	0.921	0.995	0.882	
Mock	0.073	0.065	0.063	0.063	0.066	0.068	0.067	0.069	0.066	0.067	0.071	0.072	
Mock	0.07	0.068	0.067	0.066	0.068	0.069	0.071	0.071	0.07	0.071	0.075	0.071	

Table 25.

C++	1.278	1.509	0.106	0.115	0.112	0.122	0.112	0.129	0.143	0.185	0.333	0.583	
C++	1.302	1.255	0.101	0.105	0.103	0.116	0.101	0.118	0.133	0.167	0.298	0.512	
C+	0.478	0.424	0.579	0.893	0.824	0.859	0.864	0.743	0.901	0.883	0.671	0.887	**Sheep**
C+	0.485	0.433	0.58	0.917	0.876	0.86	0.882	0.736	0.914	0.918	0.68	0.909	**3295**
C-	0.09	0.082	0.847	0.959	0.965	0.854	0.982	0.977	0.971	0.956	0.977	1.018	
C-	0.086	0.082	0.961	0.935	0.962	0.847	0.967	0.949	0.909	0.854	0.924	1.007	
Mock	0.06	0.057	1.005	0.997	0.986	0.058	0.056	0.056	0.058	0.058	0.058	0.062	
Mock	0.06	0.058	0.999	1.049	1.024	0.059	0.059	0.059	0.059	0.059	0.06	0.059	

Table 26.

C++	1.127	1.102	0.15	0.156	0.146	0.16	0.148	0.178	0.27	0.369	0.566	0.164	
C++	1.2	1.043	0.131	0.142	0.132	0.125	0.123	0.146	0.243	0.318	0.494	0.416	
C+	0.284	0.25	0.527	0.714	0.793	0.691	0.797	0.801	0.845	0.82	0.83	0.982	**Sheep**
C+	0.282	0.231	0.481	0.663	0.734	0.725	0.767	0.725	0.825	0.769	0.815	0.953	**3298**
C-	0.104	0.086	0.888	0.867	0.887	0.805	0.886	0.935	0.942	0.926	0.928	1.081	
C-	0.097	0.083	0.879	0.86	0.904	0.869	0.939	0.971	0.936	0.912	0.872	1.099	
Mock	0.062	0.057	0.857	0.887	0.056	0.056	0.057	0.057	0.059	0.058	0.061	0.061	
Mock	0.061	0.059	0.879	0.815	0.058	0.059	0.059	0.059	0.06	0.061	0.06	0.061	

Table 27. Raw data of Indirect IgM ELISA for the detection of IgM antibodies against RVFV in sheep, using three dilutions of the recombinant RVFV nucleoprotein antigen against 1:500 to 1:4000 dilutions of the HRPO conjugated detecting antibody

	RVFV rN 1:500				RVFV rN 1:1000				RVFV rN 1:2000			
C++	1.136	0.626	0.361	0.229	0.883	0.437	0.271	0.172	0.641	0.338	0.212	0.149
C++	1.203	0.607	0.356	0.218	0.802	0.402	0.254	0.164	0.617	0.326	0.204	0.145
C+	0.286	0.152	0.112	0.086	0.167	0.108	0.084	0.073	0.124	0.09	0.075	0.071
C+	0.279	0.163	0.113	0.087	0.162	0.108	0.084	0.071	0.125	0.091	0.076	0.07
C-	0.072	0.062	0.065	0.059	0.064	0.061	0.059	0.059	0.063	0.06	0.063	0.063
C-	0.074	0.062	0.062	0.059	0.064	0.061	0.06	0.059	0.063	0.061	0.06	0.061
Mock	0.065	0.058	0.059	0.058	0.06	0.059	0.061	0.058	0.061	0.059	0.059	0.06
Mock	0.066	0.06	0.06	0.059	0.063	0.061	0.06	0.059	0.063	0.061	0.06	0.061
	1:500	1:1000	1:2000	1:4000	1:500	1:1000	1:2000	1:4000	1:500	1:1000	1:2000	1:4000
	Conjugate concentration											

Table 28. Raw data of Sandwich ELISA for the detection of IgG antibodies against RVFV in sheep, using the recombinant RVFV nucleoprotein as antigen

	RVFV rN 1:1000			RVFV rN 1:2000			RVFV rN 1:4000			RVFV rN 1:8000		
C++	0.712	0.685	0.673	0.554	0.548	0.544	0.398	0.406	0.387	0.28	0.279	0.284
C++	0.693	0.66	0.667	0.52	0.519	0.503	0.361	0.371	0.354	0.257	0.258	0.271
C+	0.25	0.237	0.231	0.195	0.194	0.193	0.157	0.153	0.156	0.129	0.124	0.138
C+	0.248	0.241	0.229	0.198	0.193	0.194	0.157	0.155	0.154	0.129	0.127	0.135
C-	0.103	0.1	0.097	0.097	0.099	0.096	0.097	0.095	0.093	0.095	0.095	0.098
C-	0.102	0.096	0.097	0.097	0.095	0.097	0.095	0.094	0.092	0.093	0.095	0.096
Mock	0.083	0.078	0.078	0.078	0.077	0.078	0.078	0.078	0.078	0.079	0.078	0.096
Mock	0.086	0.081	0.081	0.081	0.083	0.083	0.081	0.079	0.079	0.075	0.083	0.081

Table 29. Raw data of IgM capture ELISA done for the detection of IgG antibodies against RVFV in livestock, using the RVFV recombinant nucleoprotein as antigen

	RVFV rN 1:1000			RVFV rN 1:2000			RVFV rN 1:4000			RVFV rN 1:8000		
C++	0.424	0.416	0.417	0.358	0.348	0.347	0.278	0.283	0.277	0.217	0.216	0.223
C++	0.403	0.416	0.406	0.353	0.356	0.347	0.274	0.286	0.288	0.216	0.219	0.218
C+	0.123	0.12	0.12	0.11	0.113	0.11	0.101	0.103	0.101	0.093	0.095	0.098
C+	0.124	0.124	0.121	0.113	0.111	0.111	0.102	0.101	0.101	0.094	0.093	0.098
C-	0.088	0.084	0.081	0.08	0.082	0.081	0.079	0.079	0.08	0.079	0.078	0.083
C-	0.088	0.082	0.08	0.081	0.083	0.079	0.081	0.08	0.081	0.079	0.079	0.083
Mock	0.092	0.087	0.083	0.087	0.082	0.082	0.083	0.082	0.082	0.083	0.083	0.081
Mock	0.088	0.089	0.086	0.088	0.086	0.088	0.086	0.085	0.086	0.088	0.089	0.09

Table 30. Raw data of IgM immune response of 10 Smithburn vaccinated sheep using a Maxisorp ELISA plate with overnight coating of RVFV rN as antigen in Carbonate/Bicarbonate buffer at 4°C

C++	1.124	1.11	0.149	0.152	0.174	0.182	0.191	0.191	0.205	0.246	0.278	0.352	
C++	1.086	1.028	0.135	0.137	0.154	0.162	0.174	0.174	0.186	0.223	0.257	0.334	
C+	0.258	0.239	0.25	0.284	0.256	0.271	0.237	0.267	0.232	0.188	0.192	0.238	
C-	0.086	0.079	0.235	0.255	0.236	0.249	0.219	0.25	0.22	0.183	0.19	0.239	Sheep 3290
C-	0.095	0.078	0.197	0.127	0.116	0.118	0.122	0.143	0.239	0.268	0.373	0.625	Sheep 3291
Mock	0.074	0.067	0.189	0.12	0.109	0.105	0.122	0.149	0.229	0.26	0.366	0.602	
	0.853	0.754	0.41	0.481	0.43	0.281	0.283	0.328	0.281	0.24	0.232	0.244	
	0.501	0.666	0.408	0.441	0.42	0.268	0.3	0.317	0.269	0.251	0.234	0.268	

Table 31.

C++	1.372	1.351	0.157	0.157	0.165	0.184	0.203	0.238	0.35	0.562	1.137	0.711
C++	1.302	1.297	0.14	0.144	0.139	0.169	0.184	0.215	0.317	0.515	1.12	0.699
C+	0.319	0.309	0.567	0.556	0.631	0.431	0.397	0.391	0.352	0.329	0.319	0.341
C+	0.305	0.303	0.57	0.531	0.597	0.429	0.354	0.383	0.326	0.318	0.309	0.33 Sheep 3292
C-	0.083	0.077	0.108	0.111	0.124	0.182	0.22	0.313	0.316	0.389	0.575	0.764 Sheep 3293
C-	0.085	0.079	0.107	0.108	0.124	0.181	0.212	0.29	0.3	0.376	0.611	0.762
Mock	0.073	0.065	0.932	0.756	0.372	0.344	0.294	0.269	0.231	0.209	0.211	0.213
Mock	0.071	0.071	0.996	0.402	0.384	0.362	0.317	0.282	0.252	0.234	0.224	0.25

Table 32.

C++	1.291	1.286	0.222	0.229	0.256	0.236	0.298	0.367	0.64	0.853	0.85	0.743
C++	1.263	1.24	0.205	0.207	0.234	0.216	0.27	0.323	0.599	0.828	0.825	0.745
C+	0.309	0.293	0.594	0.514	0.471	0.421	0.422	0.434	0.446	0.516	0.367	0.333
C+	0.305	0.294	0.559	0.495	0.448	0.321	0.389	0.366	0.378	0.5	0.338	0.329 Sheep 3297
C-	0.087	0.075	0.064	0.062	0.062	0.063	0.067	0.065	0.064	0.063	0.063	0.073
C-	0.085	0.076	0.063	0.063	0.063	0.064	0.08	0.064	0.063	0.063	0.064	0.073
Mock	0.072	0.064	0.062	0.062	0.063	0.071	0.067	0.069	0.062	0.062	0.063	0.069
Mock	0.069	0.068	0.067	0.066	0.067	0.067	0.069	0.067	0.065	0.067	0.067	0.066

Table 33.

C++	1.208	1.201	0.15	0.172	0.162	0.18	0.184	0.215	0.38	0.296	0.428	0.494
C++	1.209	1.156	0.148	0.166	0.158	0.17	0.179	0.206	0.353	0.284	0.419	0.498
C+	0.297	0.296	0.463	0.368	0.373	0.353	0.338	0.312	0.299	0.307	0.312	0.309
C-	0.1	0.103	0.455	0.368	0.379	0.364	0.346	0.312	0.273	0.3	0.303	0.304 Sheep 3296
C-	0.098	0.1	0.254	0.153	0.169	0.172	0.173	0.192	0.298	0.529	0.745	0.878 Sheep 3299
Mock	0.087	0.086	0.246	0.148	0.163	0.163	0.173	0.192	0.292	0.525	0.726	0.835
	0.766	0.632	0.649	0.642	0.496	0.414	0.384	0.379	0.373	0.326	0.347	0.327
	0.733	0.639	0.624	0.536	0.469	0.399	0.36	0.369	0.368	0.329	0.358	0.336

Table 34.

C++	1.234	1.217	0.35	0.392	0.426	0.471	0.446	0.419	0.41	0.44	0.457	0.493
C++	1.183	1.122	0.302	0.323	0.344	0.42	0.396	0.369	0.354	0.397	0.424	0.443
C+	0.291	0.267	0.418	1.346	1.162	0.89	0.906	0.769	0.829	0.824	0.755	0.796
C+	0.292	0.274	0.394	1.286	1.103	0.83	0.863	0.707	0.721	0.745	0.694	0.737
C-	0.088	0.076	0.66	0.534	0.505	0.56	0.512	0.452	0.49	0.443	0.538	0.584
C-	0.09	0.075	0.661	0.544	0.478	0.53	0.512	0.446	0.486	0.453	0.514	0.557 Sheep 3294
Mock	0.077	0.065	0.068	0.066	0.065	0.069	0.067	0.066	0.072	0.068	0.071	0.082
Mock	0.085	0.071	0.078	0.077	0.073	0.074	0.076	0.072	0.077	0.076	0.087	0.09

Table 35.

C++	1.237	1.234	0.244	0.264	0.245	0.297	0.245	0.306	0.38	0.486	0.938	1.224	
C++	1.152	1.158	0.216	0.235	0.207	0.253	0.214	0.266	0.347	0.442	0.92	1.16	
C+	0.312	0.287	0.951	0.069	1.226	0.862	1.026	0.73	0.972	1.013	0.565	0.639	
C+	0.307	0.278	0.848	0.066	1.081	0.8	0.824	0.653	0.838	0.953	0.549	0.602	
C-	0.102	0.081	0.645	0.488	0.386	0.239	0.393	0.387	0.412	0.408	0.4	0.422	
C-	0.102	0.083	0.656	0.431	0.361	0.23	0.373	0.392	0.395	0.408	0.371	0.42	Sheep 3295
Mock	0.094	0.089	0.425	0.42	0.428	0.118	0.075	0.075	0.074	0.08	0.079	0.09	
Mock	0.093	0.089	0.371	0.363	0.418	0.094	0.095	0.092	0.093	0.093	0.091	0.091	

Table 36.

C++	1.289	1.274	0.169	0.18	0.183	0.172	0.213	0.431	1.14	1.238	1.536	1.338	
C++	1.266	1.198	0.163	0.177	0.174	0.168	0.211	0.413	1.084	1.196	1.502	1.31	
C+	0.33	0.322	1.364	0.903	0.846	0.488	0.516	0.4	0.408	0.35	0.344	0.331	
C+	0.332	0.324	1.331	0.85	0.777	0.478	0.505	0.384	0.387	0.364	0.35	0.339	
C-	0.12	0.12	0.284	0.305	0.271	0.271	0.26	0.231	0.237	0.215	0.218	0.207	
C-	0.119	0.117	0.277	0.287	0.279	0.264	0.263	0.238	0.24	0.207	0.216	0.218	Sheep 3298
Mock	0.104	0.098	0.245	0.24	0.108	0.113	0.113	0.11	0.103	0.104	0.099	0.099	
Mock	0.106	0.101	0.24	0.243	0.111	0.109	0.11	0.11	0.104	0.105	0.097	0.096	

Table 37. Raw data of IgG immune response of 8 RVFV infected sheep using a Maxisorp ELISA plate with overnight coating of RVFV rN as antigen in Carbonate/Bicarbonate buffer at 4°C

C++	1.239	1.202	0.117	0.128	0.122	0.126	0.196	0.406	0.481	0.467	0.633	0.527	
C++	1.173	1.16	0.11	0.119	0.118	0.123	0.201	0.402	0.51	0.451	0.572	0.549	Sheep 6154
C+	0.352	0.356	0.655	0.586	0.646	0.701	0.763	0.908	0.936	1.127	1.232	1.267	
C+	0.36	0.352	0.652	0.578	0.683	0.698	0.759	0.905	0.922	1.102	1.203	1.136	
C-	0.115	0.115	0.114	0.131	0.147	0.138	0.167	0.223	0.564	0.565	0.792	0.987	
C-	0.116	0.112	0.109	0.122	0.137	0.127	0.16	0.216	0.577	0.561	0.808	0.989	Sheep 6962
Mock	0.095	0.096	0.862	1.111	1.123	1.01	1.146	1.092	1.158	1.293	1.258	1.318	
Mock	0.097	0.099	0.917	1.169	1.134	1.023	1.163	1.112	1.189	1.337	1.261	1.375	

Table 38.

C++	1.171	1.167	0.098	0.106	0.108	0.109	0.16	0.255	0.505	0.529	0.675	0.74	
C++	1.154	1.186	0.084	0.089	0.089	0.093	0.137	0.241	0.49	0.521	0.659	0.697	Sheep 6963
C+	0.36	0.349	0.712	0.906	0.944	1.011	0.968	1.057	1.03	1.198	1.218	1.265	
C+	0.36	0.347	0.703	0.887	0.908	0.963	0.879	1.027	0.999	1.174	1.226	1.267	
C-	0.103	0.1	0.111	0.096	0.09	0.09	0.107	0.179	0.447	0.524	0.715	0.557	
C-	0.104	0.1	0.109	0.1	0.099	0.097	0.115	0.179	0.444	0.523	0.707	0.58	Sheep 6967
Mock	0.091	0.085	0.818	0.773	0.863	0.816	0.892	0.888	0.941	1.167	1.176	1.165	
Mock	0.099	0.097	0.808	0.703	0.779	0.767	0.838	0.861	0.898	1.088	1.157	1.199	

Table 39.

C++	1.254	1.277	0.095	0.117	0.121	0.12	0.178	0.287	0.459	0.547	0.517	0.725	
C++	1.26	1.24	0.081	0.097	0.095	0.098	0.147	0.253	0.433	0.508	0.481	0.701	Sheep 6145
C+	0.387	0.375	0.608	0.796	0.628	0.65	1.002	0.77	0.942	1.163	1.226	1.321	
C+	0.391	0.364	0.607	0.784	0.6	0.643	0.986	0.743	0.905	1.171	1.217	1.303	
C-	0.101	0.097	0.073	0.088	0.09	0.096	0.116	0.259	0.38	0.479	0.784	0.629	
C-	0.102	0.098	0.077	0.093	0.092	0.098	0.117	0.245	0.376	0.471	0.783	0.642	Sheep 6537
Mock	0.084	0.076	0.709	0.739	0.818	0.883	0.939	1.024	1.094	1.207	1.25	1.348	
Mock	0.09	0.085	0.725	0.736	0.853	0.908	0.96	1.057	1.164	1.268	1.305	1.378	

Table 40.

C++	1.381	1.347	0.101	0.107	0.109	0.107	0.133	0.314	0.517	0.64	0.815	1.025	
C++	1.383	1.319	0.084	0.086	0.09	0.085	0.105	0.289	0.485	0.613	0.773	0.976	Sheep 6572
C+	0.431	0.407	1.067	1.05	1.187	1.087	1.156	1.15	1.288	1.343	1.326	1.391	
C+	0.432	0.402	1.059	1.016	1.134	1.058	1.115	1.112	1.248	1.321	1.325	1.431	
C	0.104	0.095	0.083	0.086	0.084	0.093	0.138	0.262	0.484	0.587	0.755	0.967	
C-	0.106	0.094	0.084	0.088	0.083	0.095	0.135	0.249	0.479	0.589	0.731	0.949	Sheep 6650
Mock	0.082	0.071	0.955	0.965	0.986	0.958	1.115	1.232	1.22	1.299	1.247	1.397	
Mock	0.084	0.077	0.928	0.968	0.992	0.949	1.112	1.256	1.26	1.332	1.324	1.401	

Table 41.

C++	1.378	1.416	1.486	0.989	0.845	1.466	0.597	0.822	1.493	0.578	0.771	1.357	
C++	1.401	1.343	1.487	0.95	0.827	1.482	0.566	0.809	1.492	0.554	0.717	1.319	
C+	0.464	0.45	0.861	0.852	0.063	0.063	0.063	0.063	0.064	0.064	0.068	0.078	
C+	0.457	0.454	0.867	0.837	0.063	0.063	0.063	0.063	0.065	0.065	0.068	0.084	Last 3 collections of all 8 sheep
C-	0.11	0.094	1.335	0.847	0.878	1.512	0.92	0.857	1.511	0.897	0.788	1.498	
C-	0.108	0.095	1.286	0.835	0.886	1.492	0.891	0.872	1.481	0.883	0.778	1.45	
Mock	0.084	0.07	0.813	0.817	0.07	0.068	0.07	0.069	0.071	0.067	0.069	0.075	
Mock	0.075	0.073	0.777	0.782	0.075	0.074	0.073	0.073	0.075	0.076	0.075	0.075	

Table 42. Raw data of IgM immune response of 8 RVFV infected sheep using a Maxisorp ELISA plate with overnight coating of RVFV rN as antigen in Carbonate/Bicarbonate buffer at 4°C

C++	1.19	1.165	0.16	0.228	0.213	0.208	0.895	1.781	1.875	2.133	1.879	2.08	
C++	1.155	1.119	0.163	0.228	0.218	0.216	0.855	1.608	1.705	2.053	1.839	1.984	Sheep 6154
C+	0.294	0.308	1.783	1.96	1.946	1.598	1.733	0.983	0.807	0.56	0.391	0.434	
C+	0.299	0.315	1.712	1.958	1.93	1.531	1.682	0.906	0.744	0.502	0.376	0.416	
C-	0.116	0.127	0.179	0.253	0.266	0.201	0.457	0.916	1.642	1.732	1.614	1.683	
C-	0.115	0.122	0.165	0.233	0.277	0.222	0.495	0.956	1.626	1.734	1.624	1.708	Sheep 6962
Mock	0.101	0.111	1.363	1.487	1.376	1.034	1.099	0.631	0.511	0.402	0.354	0.377	
Mock	0.103	0.104	1.441	1.613	1.49	1.113	1.191	0.649	0.503	0.377	0.342	0.347	

Table 43.

C++	1.191	1.161	0.136	0.14	0.141	0.173	0.609	1.314	2.163	2.202	1.954	2.179	
C++	1.135	1.079	0.122	0.134	0.127	0.16	0.491	1.14	2.015	2.065	1.788	2.03	Sheep 6963
C+	0.288	0.275	1.631	1.749	1.607	1.129	1.377	0.765	0.666	0.31	0.242	0.255	
C+	0.289	0.274	1.549	1.666	1.471	1.046	1.237	0.735	0.646	0.295	0.235	0.252	
C-	0.102	0.097	0.157	0.14	0.146	0.155	0.316	0.894	1.292	1.668	1.839	1.642	
C-	0.105	0.097	0.155	0.162	0.151	0.178	0.374	0.95	1.299	1.673	1.827	1.613	Sheep 6967
Mock	0.092	0.087	1.189	1.251	1.184	1.05	1.083	0.574	0.498	0.359	0.321	0.34	
Mock	0.086	0.095	1.285	1.34	1.282	1.186	1.134	0.57	0.543	0.373	0.345	0.354	

Table 44.

C++	1.225	1.18	0.179	0.305	0.239	0.241	0.571	1.367	2.04	2.157	1.917	2.256	
C++	1.167	1.097	0.148	0.221	0.179	0.19	0.442	1.111	1.821	1.935	1.676	2.089	Sheep 6145
C+	0.286	0.268	1.772	1.908	1.779	1.672	1.49	1.04	0.805	0.429	0.387	0.439	
C+	0.283	0.264	1.687	1.877	1.714	1.555	1.378	1.022	0.772	0.443	0.384	0.442	
C-	0.098	0.085	0.102	0.184	0.163	0.197	0.369	1.128	1.623	1.875	1.62	1.912	
C-	0.094	0.086	0.1	0.183	0.164	0.19	0.386	1.252	1.643	1.942	1.655	1.957	Sheep 6537
Mock	0.091	0.079	1.777	1.733	1.613	1.431	1.499	0.996	0.693	0.419	0.35	0.327	
Mock	0.088	0.09	1.856	1.812	1.716	1.548	1.611	1.081	0.771	0.464	0.395	0.378	

Table 45.

C++	1.196	1.154	0.131	0.142	0.151	0.158	0.382	1.461	2.02	2.146	1.918	1.995	
C++	1.166	1.105	0.122	0.127	0.136	0.146	0.352	1.443	1.906	2.014	1.825	1.968	Sheep 6572
C+	0.284	0.281	1.796	1.824	1.523	1.567	1.293	0.771	0.569	0.334	0.288	0.309	
C+	0.284	0.274	1.743	1.756	1.389	1.445	1.219	0.733	0.541	0.318	0.28	0.281	
C-	0.087	0.079	0.118	0.134	0.135	0.177	0.455	1.056	1.811	1.836	1.652	1.793	
C-	0.086	0.079	0.113	0.137	0.135	0.171	0.476	1.077	1.788	1.851	1.723	1.821	Sheep 6650
Mock	0.079	0.071	1.665	1.68	1.448	1.297	1.394	0.756	0.529	0.408	0.364	0.296	
Mock	0.082	0.079	1.752	1.761	1.529	1.4	1.493	0.793	0.56	0.415	0.387	0.294	

Table 46.

C++	1.313	1.292	0.554	0.169	0.314	0.416	0.201	0.141	0.277	0.179	0.116	0.318	
C++	1.303	1.269	0.503	0.16	0.31	0.35	0.194	0.127	0.257	0.169	0.106	0.304	
C+	0.307	0.301	0.129	0.109	0.067	0.066	0.066	0.066	0.067	0.066	0.069	0.079	
C+	0.304	0.3	0.126	0.107	0.065	0.066	0.065	0.067	0.067	0.066	0.068	0.077	Last 3 collections of all 8 sheep
C-	0.096	0.079	0.373	0.114	0.113	0.395	0.221	0.147	0.285	0.139	0.165	0.337	
C-	0.088	0.079	0.343	0.113	0.11	0.363	0.223	0.155	0.306	0.144	0.178	0.366	
Mock	0.074	0.066	0.112	0.113	0.067	0.068	0.068	0.068	0.066	0.066	0.067	0.09	
Mock	0.071	0.072	0.119	0.123	0.075	0.076	0.081	0.075	0.073	0.073	0.074	0.074	

Table 47.

C++	1.535	1.472	0.355	0.221	0.146	0.101	0.079	0.067	0.061	0.059	0.058	0.06	V1
C++	1.469	1.349	0.343	0.215	0.142	0.102	0.079	0.066	0.062	0.06	0.058	0.059	
C+	0.484	0.428	1.284	0.923	0.65	0.403	0.244	0.152	0.104	0.08	0.067	0.066	V11
C+	0.281	0.398	1.269	0.923	0.629	0.384	0.234	0.151	0.101	0.079	0.067	0.064	
C-	0.104	0.093	0.373	0.233	0.16	0.113	0.084	0.07	0.063	0.059	0.057	0.061	V21
C-	0.097	0.091	0.316	0.2	0.139	0.101	0.079	0.067	0.061	0.058	0.058	0.059	
Mock	0.059	0.055	1.085	0.739	0.474	0.292	0.182	0.121	0.088	0.073	0.065	0.063	V61
Mock	0.059	0.061	1.07	0.71	0.441	0.276	0.173	0.116	0.089	0.074	0.066	0.062	

Table 48.

C++	1.479	1.364	1.149	0.781	0.495	0.301	0.181	0.126	0.092	0.076	0.067	0.065	V71
C++	1.499	1.338	1.096	0.734	0.447	0.278	0.172	0.114	0.088	0.072	0.064	0.062	
C+	0.466	0.43	0.432	0.266	0.18	0.122	0.089	0.073	0.064	0.06	0.058	0.063	V131
C+	0.439	0.401	0.418	0.267	0.174	0.118	0.089	0.072	0.064	0.06	0.059	0.06	
C-	0.095	0.085	1.217	0.826	0.538	0.339	0.205	0.131	0.095	0.076	0.066	0.065	V152
C-	0.095	0.088	1.188	0.831	0.519	0.323	0.193	0.131	0.094	0.076	0.067	0.065	
Mock	0.058	0.055	0.889	0.595	0.367	0.226	0.142	0.1	0.079	0.068	0.061	0.061	V161
Mock	0.057	0.057	0.888	0.613	0.382	0.231	0.148	0.106	0.082	0.07	0.064	0.061	

Table 49.

1.223	1.244	1.462	0.991	0.631	0.396	0.237	0.147	0.102	0.081	0.073	0.065	1147
1.293	1.283	1.441	0.968	0.616	0.367	0.219	0.136	0.098	0.077	0.066	0.063	
0.476	0.442	1.749	1.104	0.648	0.38	0.225	0.14	0.1	0.077	0.068	0.068	1345
0.482	0.448	1.7	1.051	0.653	0.373	0.218	0.137	0.096	0.076	0.067	0.064	
0.111	0.096	0.504	0.307	0.193	0.133	0.097	0.076	0.067	0.06	0.059	0.063	1376
0.102	0.092	0.483	0.301	0.186	0.125	0.093	0.075	0.064	0.06	0.059	0.06	
0.057	0.055	1.698	0.918	0.519	0.298	0.172	0.112	0.084	0.069	0.062	0.061	1435
0.062	0.058	1.232	0.705	0.428	0.243	0.148	0.102	0.079	0.068	0.064	0.061	

Table 50.

1.504	1.373	0.961	0.542	0.321	0.195	0.133	0.099	0.082	0.07	0.064	0.065	1453
1.39	1.296	0.958	0.509	0.294	0.178	0.12	0.089	0.072	0.065	0.063	0.061	
0.535	0.456	1.919	1.188	0.685	0.381	0.23	0.144	0.099	0.079	0.069	0.067	1454
0.522	0.455	1.96	1.183	0.693	0.392	0.234	0.152	0.103	0.08	0.068	0.068	
0.114	0.099	1.548	0.872	0.494	0.285	0.19	0.118	0.089	0.073	0.066	0.067	1464
0.11	0.092	1.391	0.809	0.438	0.252	0.162	0.112	0.085	0.07	0.063	0.065	
0.064	0.058	2.761	2.038	1.339	0.769	0.44	0.256	0.159	0.106	0.082	0.072	1467
0.06	0.058	2.555	1.805	1.208	0.694	0.394	0.238	0.152	0.107	0.083	0.072	

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LIST OF ABBREVIATIONS

ABTS = 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)

AcMNPV = *Autographa californica* multiple nuclear polyhedrosis virus

Ag++ = strong positive antigen control

Ag- = negative antigen control

amp = ampicillin

C++ = strong positive serum control

C+ = low positive serum control

C- = negative serum control

DMSO = Dimethyl sulfoxide

DNA = Deoxy-Ribonucleic acid

d.p.i. = days post infection

ddH_2O = double distilled water

dNTP = deoxyribonucleotide triphosphate

DTT = 1,4-Dithiothreitol

EDTA = ethylenediaminetetraacetic acid

ELISA = Enzyme Linked Immunosorbent Assay

GTE = Glucose Tris EDTA buffer

HRPO = Horseradish peroxidase

IPTG = Isopropyl-β-D-1-thiogalactopyranoside

KAc = Potassium acetate

kDa = kilo Dalton

LB = Luria Bertani

LBON = LB media deficient of NaCl

N = nucleoprotein

Ni = nickel

NICD = National Institute for Communicable Disease

NSs = non-structural protein from the RVFV S segment

OD = Optical density

ORF = Open reading frame

OVI = Onderstepoort Veterinary Institute

PBS = Phosphate buffered saline

PCR = Polymerase Chain Reaction

PP = percentage positivity

rN = recombinant nucleoprotein

RNA = Ribonucleic acid

RT-PCR = Reverse Transcriptase PCR

RVFV = Rift Valley fever virus

SDS = sodium dodecyl sulphate

SDS-PAGE = SDS-Polyacrylamide gel electrophoresis

SPU = Special Pathogens Unit

TAE = Tris Acetate EDTA

TBE = Tris Borate EDTA

$TCID_{50}$ = Tissue culture infectious dosage at 50%

TE = tris-EDTA

TEV = Tobacco etch virus

UV = ultraviolet

vRNA = viral RNA

vcRNA = viral complementary RNA

APPENDIX 3

Title and abstract of paper published in the Journal of Virological Methods, 140 (2007) 106-114 (accepted 8 November 2006)

Preparation and evaluation of a recombinant Rift Valley fever virus N protein for the detection of IgG and IgM antibodies in humans and animals by indirect ELISA

Petrus Jansen van Vuren[a], Abraham C. Potgieter[b], Janusz T. Paweska[c]* Albie A. van Dijk[a]

a North-West University, Potchefstroom 2520, South Africa
b Biochemistry Division, Onderstepoort Veterinary Institute, Onderstepoort 0110, South Africa
c Special Pathogens Unit, National Institute for Communicable Diseases, Private Bag X4, Sandringham 2131, South Africa

Abstract

This paper describes the cloning, sequencing and bacterial expression of the N protein of the Rift Valley fever virus (RVFV) ZIM688/78 isolate and its evaluation in indirect ELISAs (I-ELISA) for the detection of IgM and IgG antibodies in human and sheep sera. Sera used for the evaluation were from 106 laboratory workers immunized with an inactivated RVF vaccine, 16 RVF patients, 168 serial bleeds from 8 sheep experimentally infected with wild type RVFV and 210 serial bleeds from 10 sheep vaccinated with the live-attenuated Smithburn RVFV strain. All human and animal sera that tested positive in the virus neutralisation (VN) test were also positive in the IgG I-ELISA. There was a high correlation ($R2 = 0.8571$) between VN titres and IgG I-ELISA readings in human vaccinees. In experimentally infected sheep IgG antibodies were detected from day 4 to 5 post infection onwards and IgM antibodies from day 3 to 4. The IgG I-ELISA was more sensitive than VN and haemagglutinationinhibition tests in detecting the early immune response in experimentally infected sheep. The I-ELISAs demonstrated that the IgG and IgM response to the Smithburn vaccine strain was slower and the levels of antibodies induced markedly lower than to wild type RVFV infection.

Keywords: Rift Valley fever virus; recombinant N protein, IgM and IgG indirect ELISA, humans, sheep.
*Corresponding author. Tel.: +27 11 3866382; Fax: +27 11 8823741.
E-mail address: januszp@nicd.ac.za (J.T. Paweska)

APPENDIX 4

Title and abstract of presentation held at the Molecular and Cell Biology Group Symposium, 5 October 2006, hosted by the University of the Witwatersrand Medical School at the Johannesburg Hospital Auditorium

Production and evaluation of a recombinant Rift Valley fever virus nucleocapsid protein for the detection of IgG and IgM antibodies in humans and animals by indirect ELISA

Jansen van Vuren, P.[a,c,*], Potgieter, A.C.[b], Paweska, J.T.[c], Van Dijk, A.A.[a]

[a] North-West University, Private Bag X6001, Potchefstroom 2520, South Africa
[b] Biochemistry Division, Onderstepoort Veterinary Institute, Onderstepoort 0110, South Africa
[c] Special Pathogens Unit, National Institute for Communicable Diseases, Sandringham 2131, South Africa.
[*] Presenting author

Abstract

The objective of this study was to clone and express the nucleocapsid (N) protein of Rift Valley fever virus (RVFV) and evaluate its applicability as a diagnostic antigen in indirect ELISA (I-ELISA). The gene encoding the RVFV N protein was amplified using RT-PCR and the amplicon subsequently sequenced and cloned into the bacterial expression vector pET32(a)+ using specific restriction enzyme sites added to the amplicon by the primers. The confirmed plasmid was used to transform competent Origami(DE3) cells in which expression was optimized. The resultant recombinant N fusion protein containing a 6x histidine tag was purified using metal affinity chromatography and used to coat immunoplates. Optimum conditions for the I-ELISA were determined by standard checkerboard titrations using a panel of control sera. Field sera tested were taken from 106 laboratory workers immunized against RVFV, 16 RVF patients, 168 serial bleeds from 8 sheep experimentally infected with wild type RVF virus and 210 serial bleeds from 10 sheep vaccinated with the live-attenuated Smithburn strain of the virus. The I-ELISA was able to readily to detect both IgG and IgM antibodies in human and animal sera. There was a very high correlation between the I-ELISA and virus neutralization (VN) test results in human vaccinees (R^2 = 0.857). Compared to VN and haemagglutination-inhibition tests results, the I-ELISA was more sensitive in detecting the early immune response in experimentally infected sheep. We conclude that the I-ELISA based on recombinant N protein has the potential to replace traditional diagnostic methods which are unable to distinguish between different immunoglobulin classes wherein the preparation of the antigen poses a biohazard health risk to laboratory personnel.

APPENDIX 5

Title and abstract of poster presentated at the National Institute for Communicable Disease academic day, 28 November 2006.

Recombinant Rift Valley fever indirect ELISA for the detection of IgG antibodies in humans

Introduction:
Rift Valley fever (RVF) is an important zoonotic and a potential biothreat agent. Current serological techniques require manipulation of live RVF virus either during the production of antigen stocks and/or when the tests are carried out. These laboratory procedures comprise a biohazard.

Aims:
The objective of this study was to validate an indirect ELISA (I-ELISA) based on recombinant nucleocapsid (N) protein of RVF virus for the detection of specific IgG antibody in human sera.

Methods:
Optimal conditions for the I-ELISA were determined by standard checkerboard titration. Validation data sets derived from testing field-collected sera from Africa (n = 2551) were dichotomised according to the results of a virus neutralisation test. ELISA data were expressed as a percentage of high-positive control serum (PP). A cut-off value at 95 % accuracy level was optimised using the misclassification cost term option of the two-graph receiver operating characteristic analysis.

Results:
There was no evidence for excessive intra- and inter-plate variation within and between routine runs of the assay. At a cut-off of 29.4 PP the sensitivity of the I-ELISA was 99.65% and specificity 99.86%. Other estimates of combined measures of diagnostic accuracy were also high.

Conclusion:
The indirect ELISA based on recombinant N protein of RVF virus is safe, robust and highly accurate, therefore, it has the potential to replace traditional diagnostic methods which pose health risks necessitating their use being restricted to high containment facilities outside RVF endemic areas.

Presenting Author:
Janusz Paweska
Special Pathogens Unit - National Institute for Communicable Diseases

Other Authors:
1. Petrus Jansen van Vuren
2. Albie van Dijk
3. Robert Swanepoel