Preparation of recombinant antigen for serological detection of African hantaviruses

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M. Med. Sc

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Dissertation submitted in fulfilment of the requirements for the degree M.Med.Sc. Virology in the Department of Medical Microbiology and Virology, Faculty of Health Sciences, University of the Free State, Bloemfontein

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Abbreviations

ANDV	Andes virus
ARDS	adult respiratory distress syndrome
BHK-21	baby hamster kidney cells
cRNA	complementary-sense RNA
DOBV	Dobrava-Belgrade virus
DMEM	Dulbeccos' minimum essential media
ELISA	enzyme-linked immunosorbent assay
FBS	foetal bovine serum
GFP	green fluorescent protein
GPC	glycoprotein precursor
HCPS	hantavirus cardio pulmonary syndrome
HFRS	hemorrhagic fever with renal syndrome
HNTV	Hantaan virus
IFA	indirect immunofluorescence assay
KHF	Korean hemorrhagic fever
L	large segment
M	medium segment
MOUV	Mouyassué virus
NCR	non-coding region
NE	nephropathia epidemica
NEAA	non-essential amino acids
NHLS	National Health Laboratory Service
NP	nucleocapsid protein

NS	non-structural proteins
OD	optical density
ORF	open reading frame
PRNT	plaque reduction neutralization tests
PUUV	Puumala virus
RdRp	RNA-dependent RNA polymerase
RNP	ribonucleoprotein
RT-PCR	reverse transcription polymerase chain reaction
S	small segment
SANGV	Sangassou virus
SNV	Sin-nombre virus
SD	standard deviation
SDS	sodium dodecyl sulphate
TAE	Tris-acetate-EDTA
TPMV	Thottapalaym virus
VEGFA	vascular endothelial growth factor A
vRNA	virus-encoded RNA

Declaration

I, Deborah Damane, certify that the dissertation hereby submitted for the M. Med.Sc Virology qualification at the University of the Free State is my independent effort and has not previously been submitted for a qualification at another university/faculty. I furthermore waive copyright of the dissertation in favour of the University of the Free State.

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Abstract

Unlike other members of the *Bunyaviridae* family, hantaviruses are transmitted to humans through direct exposure or inhalation of virus contaminated urine or droppings from their reservoir hosts. Hantaviruses were first discovered in 1976 with the identification of Hantaan virus (HNTV) from the reservoir *Apodemus agarius* in Asia and later in North America. In 2006, Sangassou virus (SANGV) was the first to be isolated in Africa in the African house mouse, *Hylomyscus sinus* and subsequently followed by the identification of ten more African hantaviruses in both rodent and insectivore hosts through reverse transcription polymerase chain reaction (RT-PCR) and immunofluorescence assay (IFA). Hantaviruses are a public health concern with annual cases of disease reported to be approximately 200,000 per year, with most cases reported in Asia. In Africa, disease associated with hantaviruses is not well defined.

Culturing the virus and preparing reagents using native virus requires the use of biosafety level (BSL) 3 or 4 laboratories limiting the number of facilities with capability to prepare serological assays. Hence, the use of recombinant antigens that are safe to use in a BSL 1 laboratory that have application as serological tools for surveillance are required.

The aim of the study was to develop serological assays to test for antibodies against hantaviruses in human serum samples collected in the Free State, South Africa using a recombinant nucleocapsid protein (NP) of SANGV as a representative of African hantaviruses. Transiently transfected cells were used to prepare antigen slides for IFA and expressed protein was used in an in-house enzyme linked immunosorbent assay (ELISA). In-house assays and commercially available ELISA kits were used to screen human serum samples.

There are limited seroprevalence studies performed in Africa to detect IgG antibodies against hantaviruses in humans and no commercial serological assays are available using an African antigen. Hence, it was considered that the preparation of a recombinant African hantavirus antigen based on SANGV could have application in serological surveillance studies.

The S gene segment of the SA14 strain of SANGV was modified and codon optimized for enhanced expression and detection. The construct was sequenced and aligned to the native S gene. It was used to transfect baby hamster kidney cells (BHK-21). Expression of a 50kDA

protein was confirmed by SDS-PAGE and Western blot assay. Antigen slides were prepared from transfected cells fixed on 12 well chamber slides. Positive controls from the commercially available ELISA kits were used in the IFA. Four of the 176 serum samples tested gave a positive test. For the in-house ELISA, protein was harvested from T75 culture flasks. The antigen was tested using positive and negative controls from the commercial ELISA kits. A suitable differentiation between positive and negative samples was not detectable despite attempts to optimize the in house ELISA. It is likely that the protein yield was insufficient for the ELISA and further attempts, beyond the scope of this project, to increase the protein yield will be investigated using a stable cell line. Commercially available ELISA kits comprising of HNTV, Dobrava (DOBV) and Puumala (PUUV) recombinant NP antigens for Europe and Asia group and Andes virus (ANDV) and SNV for the American group were used to screen acute human sera in the laboratory. Positive reactors were identified using both kits. The significance of the results is difficult to interpret as there was lack of concordance. However, it does suggest that hantaviruses are to be found in this area and that the use of a homologous antigen for serological surveillance is essential. Results confirmed some serological cross-reactivity between heterologous Asian, American and African hantaviruses and a potential application for an African hantavirus as a tool for surveillance.

Keyterms: Hantavirus, Sangassou virus, Enzyme-linked immunosorbent assay, Immunofluorescence assay, recombinant nucleocapsid protein, commercial ELISA kits, African hantaviruses

Contents

Chapter 1: Literature review

1.1 Introduction and History

Hantaviruses belong to the largest and most diverse family of RNA viruses. The *Bunyaviridae* family is comprised of over 350 described virus species, commonly referred to as bunyaviruses. Bunyaviruses are classified under five genera including *Hantavirus*, *Nairovirus*, *Phlebovirus*, *Tospovirus* and *Orthobunyavirus*. Classification into each genus was initially based on serologic cross-reactivity but has since expanded to include morphology of viruses, genome structure, phylogenetic relatedness as well as replication of the virus (Casals & Tignor, 1980; Calisher & Karabatsos, 1989; ICTV 9th report (2011), Soldan and Gonzales-Scarano, 2014; Walter and Barr, 2011).

Bunyaviruses have a worldwide distribution and are implicated in causing disease in humans and animals with potential to disrupt economic development especially in third world countries. With the exception of hantaviruses, members of the *Bunyaviridae* family are transmitted by arthropod vectors. Hantaviruses are transmitted to humans by direct exposure and inhalation of virus contaminated excreta from the reservoir hosts, which were thought to be primarily rodents but now also include shrews and insectivores. Transmission through bites rarely occurs despite the existence of a proposed model of biting and scratching as modes of transmission between the different rodent reservoirs and their spill over species that coexist in one environment (Allen et al., 2009; Soldan and Gonzales-Scarano, 2014). It is thought that the crucial factor that plays a role in the maintenance and persistence of hantaviruses is the state of the chronically infected reservoir hosts (Lee et al., 1982).

The first hantavirus, Thottapalayam virus (TPMV), was isolated from the spleen of an Indian house shrew, *Suncus murinus*, in 1964 near Vallore in India. It was presumed to be an arbovirus due to its sensitivity towards sodium deoxycholate and it was not until 1988 that the virus was classified with other bunyaviruses. TPMV was therefore not only the first hantavirus to be isolated but also the first hantavirus harboured by an insectivore as opposed to other hantaviruses that were mainly harboured by *Muridea*. TPMV has since been discovered in China, Vietnam and Nepal. It was not until 2006 that other hantaviruses

were discovered in insectivores (Soricomorpha) in other geographic regions such as some parts of Africa (Guo et al., 2013; Carey et al., 1971).

The first disease caused by hantavirus was identified in Korea in the spring of 1951. During the Korean War from 1951-1954, more than 3000 soldiers of the United Nations had symptoms of febrile illness accompanied by headache, abdominal and back pain, prostration and flushed faces. The disease, now referred to as hemorrhagic fever with renal syndrome (HFRS), formerly referred to as Korean hemorrhagic fever (KHF) is one of two disease syndromes associated with hantavirus infection in humans. Old world hantaviruses are associated with renal insufficiency whereas new world hantaviruses, found in the Americas, are the cause of a disease commonly referred to as hantavirus cardio/pulmonary syndrome (HCPS/ HPS). The distinction appears to be based on the organs targeted by the viruses. HCPS is thought to be a more lethal form of disease in hantavirus infections (Gavriloskaya et al., 1999; Lee et al., 1982; Lee et al., 2014; Oldal et al., 2014).

Decades before its discovery in modern medicine, physicians in China, Russia and Japan described a disease similar to HFRS referred to as Songo fever, nephroso-nephritis and nephropathia epidemica (NE) respectively (Myhrman, 1951). Owing to the diverse range of causative agents of illnesses closely resembling hantavirus disease, such as some bacterial infections and other viruses, its highly possible that hantaviruses might have gone unnoticed for many years. It was not until 25 years after the outbreak was described in the Korean War, that the causative agent of HFRS in UN soldiers was identified. The virus was isolated in cell culture from samples collected from the field mouse*, Apodemus* agrarius (Lee et al., 1978). Detection of the prototype hantavirus was performed by indirect immunofluorescence assay (IFA) on acetone-fixed lung sections of the reservoir hosts. The etiologic agent, Hantaan virus (HNTV) was named after the river Hantaan River in South Korea. In 1993, an unexplained adult respiratory distress syndrome (ARDS), later referred to as HCPS, was identified in the Four Corners Region of the south western United States. The etiologic agent of HCPS, isolated from the deer mouse (*Peromyscus maniculatus*), was named Sin Nombre virus (SNV) and is associated with a 50% fatality rate in human. The deer mouse, as with most reservoirs of hantaviruses, is said to have a persistent infection with neutralizing antibodies in high titers (Hjelle et al., 1994; Lee et al., 1982; Lee et al., 2014; Prescott et al., 2005).

It was not until 2006 that the first African hantavirus was identified from the blood of one of four African wood mice, *Hylomyscus simus* through reverse transcription polymerase chain reaction (RT-PCR) and later isolated in cell culture. The virus was named Sangassou virus (SANGV) after the village in Guinea, where the rodents were captured. Four major groups of hantaviruses have been identified through phylogenetic analyses. Classification of viruses into three of the four groups is based on the natural rodent hosts' subfamily i.e. Murinae-, Arvicolinae- Sigmodontinae- associated hantaviruses and the more recently added group of viruses that are carried by shrews which includes TPMV. Examples of Murinae-associated hantaviruses include HTNV and Dobrava-Belgrade virus (DOBV). An example of Arvicolinae-associated hantavirus is Puumala virus (PUUV), and SNV is an example of Sigmodontinae-associated hantavirus. Human disease caused by hantaviruses is predominant in Asia, Europe, and the Americas (Klempa et al., 2006; Klempa et al., 2012).

1.2 Virus classification

Hantaviruses belong to the family *Bunyaviridae* and are comprised of a tri-segmented, single-stranded RNA genome. The genome is encapsidated and enclosed by a lipid bilayer envelope with Gn and Gc glycoproteins forming spikes on the surface of virus particles. The RNA genome is negative sense and is comprised of three genomic segments designated small (S), medium (M) and large (L) which reflect to the relative length of the number of nucleotides (Vaheri et al., 2008; Hart and Bennett,1999). The S segment (~1800 nucleotides nt) , M segment (~3700 nt) and the L segment (~6500 nt) encode the nucleocapsid protein (NP), the glycoprotein precursor (GPC) and the RNA-dependent RNA polymerase (RdRp) respectively . The GPC is cotranslationaly cleaved to produce the Gn and Gc glycoproteins that protrude as heterodimers in a highly ordered manner on the surface of the virus particle (Gn and Gc) (Elliott et al., 1991; Spiropoulou, 2001). The RdRp mediates replication and transcription. An open reading frame (ORF) for a non-structural protein is present on the S segment of some hantaviruses carried by cricetidae rodents with an apparent function as an interferon inhibitor (Plyusnin and Morzunov, 2001). The 3' non-coding region (NCR) on the S segment of hantaviruses belonging to the same type do not differ significantly in length and nucleotide sequence but among different types of hantaviruses it differs widely, both in length and nucleotide composition which is indicative of some functional role (Hart and Bennet., 1999; Hooper et al., 2001; Jaaskelainen et al., 2007; Meisel et al., 2006; Plyusnin et al., 1996; Prescott et al., 2005).

Highly conserved genomic regions are found on the 3' and 5' termini of hantavirus gene segments. They are complementary and pairing of these regions forms panhandle structures and closed circular RNAs that are non-covalently linked which forms the hallmark of bunyaviruses. Normally genus specific, these pan-handle structures can be seen under electron microscopy and it has been postulated that they aid in viral recognition by the polymerase as well as packaging of genomic material (ICTV 9th report (2011), Kukkonen et al.,2005; Plyusnin et al., 1996). Virus particles are spherical and at times pleomorphic and range between 80-135 nm in size (Plyusnin et al., 1996; Kruger et al., 2011; Vaheri et al., 2013). The genome consists of negative sense RNA (Oldal et al., 2014). NP monomers are associated with individual viral RNAs to form structures known as ribonucleoproteins (RNPs) that are highly immunonogenic in humans and animals (Dargeviciute et al., 2002). RNPs take up a circular, flexible form and are not always packed in the same numbers in mature virions and hence account for the different size viruses as is seen under electron microscopy (Ortin and Martin-Bernito, 2015; Schmaljohn and Nichol, 2007). The hantavirus virions are comprised of 20-30% lipid, 2-7% carbohydrates and more than 50% protein (McCaughey and Hart, 2000).

1.3 Transcription and replication

Transcription of viral-encoded RNA serves as an essential starting point in the production of viral proteins. The catalytic activity of the L protein facilitates the synthesis of RNA. The association of the polymerase and infecting RNPs leads to random initiation of mRNA synthesis by cap-snatching or synthesis of replication intermediate. Viruses scavenge host cell capped nucleotides from mRNA to prime their mRNA. This is done by the endonuclease activity in the virions and associated with the L protein. This cap snatching mechanism results in mRNA that is primed with 5' 10-20 nt that are not found in vRNA. The regions for cap snatching are preferential for different viruses. Prime and realign is the term given to the transcription of mRNA of hantaviruses due to the observation of the polymerase slippage following the addition of the first few nucleotides in the nascent mRNA causing partial reiteration in the 5' sequence (Schmaljohn and Nichol., 2007; Plyusnin et al., 1996; Yao et al., 2012). Using this mechanism, a guanosine triphosphate (pppG) or a host-derived capped primer (7mGppp) for replication and transcription respectively, is hybridized to a cytosine in the 3' terminus of vRNA. Replication of hantaviruses occurs primarily in endothelial cells and macrophages (Gavriloskaya et al., 1999; Kukkonen et al., 2005).

Structural proteins are all encoded in the positive-sense RNA (cRNA) where as non-structural proteins can either be encoded for in the c- or v RNA (Schmaljohn and Nichol., 2007). Together with viruses belonging to the genus *Nairovirus,* hantaviruses are said to encode larger NPs as compared with viruses from other genera. Long repeats of 3' noncoding regions have been observed with certain hantaviruses (i.e. SNV) and it has been suggested that it may be as a result of polymerase slippage on the vRNA templates (Schmaljohn and Nichol, 2007).

The Gn and Gc glycoproteins, previously known as the G1 and G2 glycoproteins on account of their migration in polyacrylamide gels, are so called based on the amino- or carboxy-terminal coding of the proteins and are encoded for on a single ORF cRNA of the M segment. The functions of these proteins are said to be conserved among the five genera. As opposed to the coding strategies of the other segments, the L segment uses the conventional negative sense coding strategy for the RdRp. The L segment of hantaviruses are not as large as that of nairoviruses and tospoviruses that are considerably larger. They include 200 nucleotides that do not have coding information (Schmaljohn and Nichol, 2007).

1.4 Replication

Multiple cell lines are capable of being infected with hantaviruses and these include dendritic, macrophages, lymphocytes, epithelial and endothelial cells. In order for the virus to enter the cell it has to attach via interactions between the cell surface receptors (Gn and Gc spikes). On both these proteins there are sites for neutralization as well as hemagglutination-inhibition suggesting that they can be used for attachment to cells. β1 and β3 integrins have been shown to be the host cell receptors responsible for attachment, migration and transduction of intracellular signals for both infection with non-pathogenic and pathogenic hantaviruses. HNTV was shown to enter the cytoplasm of infected cells by receptor-mediated endocytosis using clathrin-coated vesicles. Other studies have reported that hantaviruses are capable of infecting cells without integrin receptors suggestive that other host receptors exist (Mou et al., 2006). It has been shown, using a confocal microscope, that the viral proteins co-localize with clathrin. Although it was postulated that Gc mediates fusion, association of the two proteins (Gn and Gc) is necessary for membrane fusion (Markotic et al., 2007; Prescott et al., 2005; Raftery et al., 2002; Schmaljohn and Nichol., 2007; Spiropoulou, 2001).

Following the disassembly of the clathrin-coated vesicle, the vesicle harbouring the virion enters early endosome that matures into a late endosome with an acidic pH (Schmaljohn and Nichol., 2007; Vaheri et al., 2013). An acidic environment is required to facilitate infectivity of the virus (Schmaljohn and Nichol., 2007). Uncoating leads to the release of the viral genome, and RNPs. Initial transcription of the vRNA into mRNA takes place either at location of release or in the endoplasmic reticulum-golgi intermediate compartment. This process begins with association of the virion to the L protein or RdRp and the three viral RNPs. It has been suggested that the NP aids initiation of transcription and replication by remaining attached to the 5‘ termini and freeing the 3’ panhandle structure and by acting as a chaperone in creating stable structures in the RNA. The L and NPs of hantaviruses localize in the perinuclear region of the cell and free RNA cannot serve as template for transcription. The 3’ and 5’ non-translated complementary regions are genome promoters and newly formed virus buds into the cis-golgi where they are the transported for release through the plasma membrane (Vaheri et al., 2013; Schmaljohn and Nichol, 2007).

For most bunyaviruses no U repeats for signal termination of transcription are observed and hence, as a result, an mRNA segment with an absence of polyadenylation is produced. However, within the hantavirus genome a U-rich region has been identified in the M mRNA segment. L and S gene segments are all truncated at the 3’ termini by an approximated number of 100 nucleotides. The switch from primed mRNA to replication of the genome is not well understood but it has been suggested that the RdRp is responsible, either working alone or with cellular factors or other undefined viral factors (Schmaljohn and Nichol., 2007).

Among its many functions, the NP protects the RNA from degradation by interacting with the vRNA at the 5’ end through the pan-handle structures. The NP of hantaviruses form stable trimeric structures that are thought to assemble themselves on the viral genome and interacts further to eventually encapsidate the entire genome. This has been predicted by the formation of coils at the N-termini of the proteins (Schmaljohn and Nichol, 2007; Vaheri et al., 2013). The NP and RdRp of hantaviruses are thought to be responsible for sequestering the caps of host mRNA to act as primers as a prerequisite for efficient translation in eukaryotic cells from cellular processing (P) bodies suggesting a direct involvement of NP in virus replication (Mir et al., 2008). In addition, there is an overlapping region of the small gene segment that encodes for a non-structural protein (NSs) in some hantaviruses carried by rodents from the *Cricetidae* family. The NSs is thought to play a role

in inhibiting activity of interferon (IFN) promoters in IFN-competent cells infected with TULV thereby suggestive that it counteracts the hosts innate immune response (Virtanen et al., 2007). The NP of certain viruses including measles virus, influenza and CCHFV have been seen to be interact with actin for transportation of virus particles to the cell surface, involvement of viral RNA synthesis as well as NP localization (Gupta et al., 1998; Moyer et al., 1990; Andersson et al., 2004). At least some of the hantaviruses, such as Black Creek Canal virus, use the NP to bind to and co localize with actin filaments and this is important for release of viruses as disruption of the filaments led to viral RNPs not being transported to the plasma membrane (Ravkov et al., 1998). Whatever the role of this protein, the NP of hantaviruses is most abundantly expressed in the cytoplasm of infected cells and considered an immunodominant antigen.

The M segment gene products have a 4-7% content of cysteine and their position among related viruses is said to be conserved. Predicted transmembrane regions of the polyproteins are varied with hydrophobic sequences at the carboxy-termini indicative of membrane anchor regions. These transmembrane proteins within this family are said to posses asparagines-linked oligosaccharides and in particular mannose sugars in hantaviruses. This is indicative of incomplete processing of the proteins in the golgi membrane (Schmaljohn and Nichol., 2007). Maturation of viruses takes place through their budding off of the smooth membranes of the golgi and for some hantaviruses from plasma membranes (Schmaljohn and Nichol., 2007).

The target for the expression of Gn and Gc proteins is the golgi membrane in the absence of other viral components. In most members of the family, when individually expressed, Gn will move out of the ER while Gc remains, however for hantaviruses both individually expressed proteins are retained within the ER. Studies have shown that complexing of the proteins, especially that of the Gc to Gn helps with transportation of the proteins to the golgi (Dyde etal., 2005; Ruusala et al., 1992; Shi and Eliiot, 2002). Assembly of viruses occurs in the cytoplasm and cytoplasmic inclusions with excessiveRNPs . Vesicles derived from the golgi membrane buds with the cellular plasma membrane for the release of the virus (Schmaljohn and Nichol, 2007).

1.5 Reservoir hosts, transmission and epidemiology of the viruses

An important ecological factor that distinguishes hantaviruses from other members of the *Bunyaviridae* family is the observation that they do not posses any arthropod vectors

(Kruger et al., 2011). Instead they are found to be hosted primarily by rodents and insectivores (moles and shrews) and more recently by bats in Côte d'Ivoire, West Africa (Calisher et al., 2005; Kruger et al., 2011; Sumibcay et al., 2012). Due to the fact that hantaviruses can be transmitted to humans by one or more closely related rodents or insectivores through aerosolized host excreta, limitation in their geographic range exists but does not rule out the possibility of the non-determined areas where possible reservoirs may exist (Klempa et al., 2012; Kruger et al., 2011).

Habitat-based models help us understand how direct transmission of a pathogen occurs between species that coexist within the same environment and in which one species is a carrier of a pathogen and the other a naïve species. When a habitat overlaps and there is contact between species there is greater opportunity for a pathogen to be transmitted, particularly when there is competitive usage of a food or water source, thereby spreading the virus in the population. Although spill over infections are not fully understood, it has been proposed that they could play an important role in the evolution of pathogens (Allen et al., 2009). Interspecies-interactions create such opportunities for the transmission of pathogens (Allen et al., 2009).

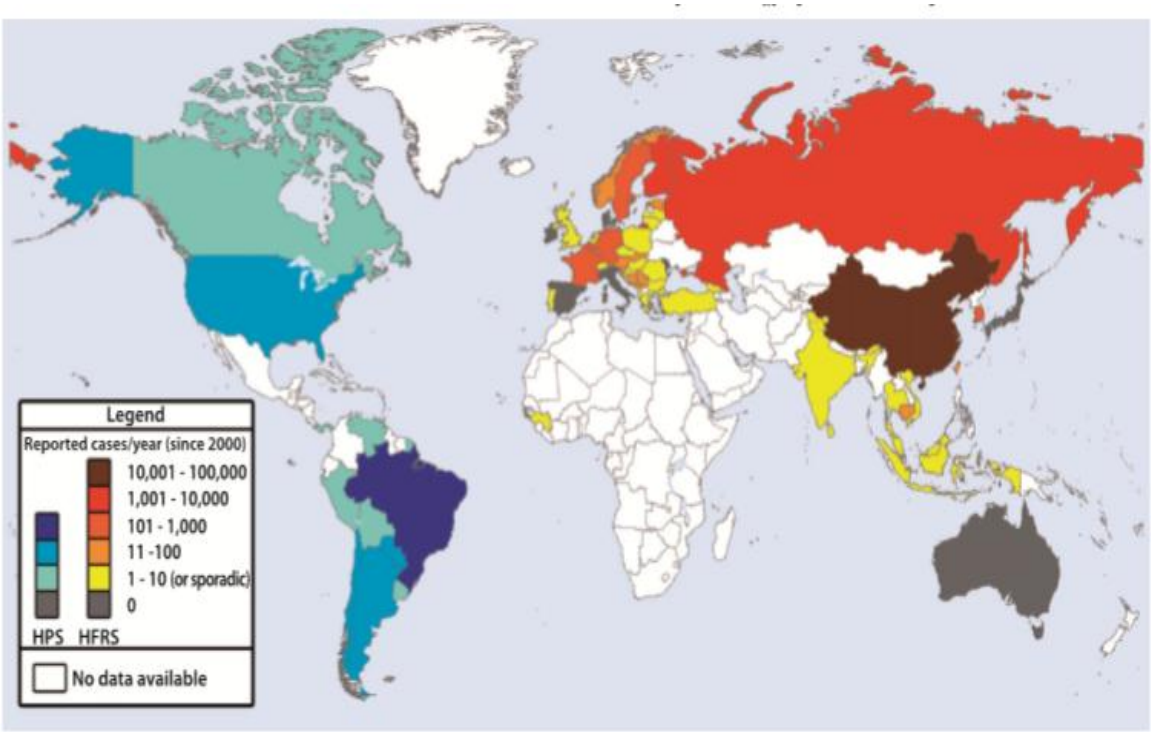

Figure 1: Geographical distribution of hantaviral infections/ year since the year 2000. Only national/ internationally reported laboratory confirmed cases are included. Imported cases are excluded. Source: Watson et al., 2014.

Hantaviruses are found throughout the world with annual cases of HFRS reported per year to be approximately 10,000 - 100,000 since 2000, with most cases reported in China, as depicted in Figure 1. Seoul virus (SEOV) which causes a mild or moderate disease and the prototype hantavirus, HNTV which causes a more severe form of HFRS, are both found in Asia. DOBV and PUUV are found in south-eastern Europe and central and northern Europe respectively. PUUV is responsible for a milder form of disease often referred to as epidemic nephropathy or NE and is most prevalent in Germany. There are some indications of circulating, non-characterized hantaviruses in Europe (Dargeviciute et al., 2002; Mattar et al., 2015; Schwarz et al., 2009; Watson et al., 2014).

It has been observed that the reservoir hosts do not show any signs or symptoms of disease but are chronically infected with persistent viremia and therefore are likely to play an important role in the maintenance and spread of the virus to humans (Allen et al., 2009; Lee et al., 1982). These chronically infected rodents however may not survive well in the winter as recent studies have indicated (Ramsden et al., 2009). PUUV, carried by the bank vole is the only human pathogen in Finland (Vaheri et al., 2014). SNV is a hantavirus carried by the deer mouse *Peromyscus maniculatus* in the four corners region of the south western United states, New Mexico, Colorado and Arizona border. SNV is the prototype of the causative agents of HCPS which caused ARDS during the outbreak in 1993. This virus accounts for more than 250 cases annually in North America alone (Bharadwaj et al., 2000; Nichol et al., 1993; Prescott et al., 2005).

Previous studies in the discovery of hantaviruses have implicated rodents as the principal hosts and it was not until their discovery in other small mammals such as shrews and moles that the range of reservoir hosts for hantaviruses became a question of interest in obtaining a true reflection of the transmission of these pathogens in Africa and elsewhere in the world (Weiss et al., 2012). TPMV is a hantavirus that was discovered in 1964 in southern India. The virus was isolated in an Asian house shrew and classified according to ultrastructural features and genetic similarities with other hantaviruses. Genetic variability of the S segment, placed TPMV as an outgroup from the other hantaviruses that are grouped into murid, arvicolid, and sigmodontine associated groups. It does not cross-neutralize in plaque reduction neutralization tests (PRNT) (Song et al., 2007).

Viral antigens have been detected in secretory cells of the salivary glands from patients with Andes virus (ANDV). Transmission of ANDV from person-to-person has been reported

(Hardestam et al., 2008) and the risk of transmission has been associated with close contact with the index case. Although viral antigens have been found in the saliva of infected individuals, the route of transmission is not well understood. It may be speculated, however, that transmission by saliva from human to human is possible due to the infectivity of the virus after treatment of cells with saliva prior to infecting with virus. It has been suggested that a window of opportunity occurs for infectious particles to be secreted in the saliva of infected persons and poses a risk of transmission to others from the time the person is infected to the time the patient has sero-converted (Hardestam et al., 2008).

1.6 Hantaviruses in Africa

The search for hantaviruses in Africa intensified after the discovery of the indigenous virus SANGV in 2006 (Klempa et al., 2006, 2012). Since then nine additional hantaviruses have been identified in Africa through RT-PCR. SANGV remains the only African hantavirus to be isolated in cell culture. A total of ten indigenous African hantaviruses have been identified to date in a number of reservoirs including rodents, shrews and more recently in bat species. Two strains of SANGV, SA14 and SA22, have been identified in African wood mice as can be seen in Table 1 (; Witkowski et al., 2014). SANGV was identified using a nested RT-PCR with degenerate primers to amplify RNA from the blood of captured rodents (Klempa et al., 2006). Two additional hantaviruses were subsequently identified, Kilimanjaro virus (KILV) and Uluguru (ULUV) using archived frozen liver tissues from rodents captured in Tanzania. Species identification was verified by analysis of the mitochondrial DNA cytochrome b gene (Kang et al., 2014). Complete or partial sequences of each segment belonging to KILV and ULUV were compared to other hantaviruses representing rodent- or Soricomorph-borne hantaviruses and were shown to form lineages separate from the rodent borne viruses using phylogenetic analysis. A common ancestry with TPMV and Imjin virus, both of Asian origin, was shown to exist for the two viruses identified in Africa (Kang et al., 2014).

Bats (order Chiroptera) have also been shown to harbour hantaviruses in West Africa. Mouyassué virus (MOUV) was detected in liver tissue fixed in ethanol from banana pipistrelles (Neoromicia nanus) in 2011 in Mouyassué village in Côte d'Ivoire. Bats are abundant, geographically dispersed and diverse. They have been implicated as host reservoirs of many pathogens including over 60 viruses from a range of different viral

families that include *Filo-, Toga-, Orthomyxo-*, and *Bunyaviridae* families (Calisher et al., 2006; Sumibcay et al., 2012).

Serological studies carried out in Guinea in a cohort of human subjects suggest that hantaviruses are capable of infecting and causing disease in humans. Findings reported seroprevalence rates of 4.4% and 1.2% for one cohort group that was comprised of patients with fever of unknown origin and human serum samples from a Lassa fever survey respectively (Klempa et al., 2010). An additional study conducted in Upper Guinea also showed seroprevalence of 1.2% (Witkowski et al., 2014). In South Africa, in the Western Cape province, patient samples were obtained from the National Health Laboratory Service (NHLS) laboratories in Paarl, Vredendal, Tygerberg and Oudtshoorn. The samples were collected between 2010 to 2012 after routine diagnostic testing and showed seroprevalence of 1.0% (Klempa et al., 2010; 2013; Witkowski et al., 2014).

Table 1: Indigenous African hantaviruses, the species from which they were identified from and the location where they were captured.

VIRUS	SPECIES (RESERVIOR HOST)	LOCATION
1. Sangassou virus (SANGV) SA14 and SA22 (Klempa et al., 2006; 2012)	African wood mouse (*Hylomyscus simus)*	Guinea
2. Tanganya virus (TGNV) (Klempa et al., 2007)	Therese's shrew *(Crocidura theresae)*	Guinea
3. Azagny (AZGV) (Kang et al., 2011)	West African pygmy shrew *(Crocidura obscurior)*	Cóte d'Ivore
4. Bowė virus (BOWV) (Gu et al., 2013)	Doucet's musk shrew *(Crocidura douceti)*	South West Guinea
5. Tigray virus (TIGV) Meheretu et al., 2012	Ethiopian white-footed mice *(Stenocephalemys albipes)*	Ethiopia
6. Magboi virus (MGBV) (Weiss et al., 2012)	Slit-faced bat *Nycteris hispida*	Seirra Leone
7. Mouyassuė virus (MOUV) (Sumibcay et al., 2012)	Banana pipistrelle *(Neoromicia nanus)*	Cóte d'Ivore
8. Kilimanjaro virus (KILV) (Kang et al., 2014	Kilimanjaro mouse shrews (*Myosorex zinki*)	Tanzania
9. Uluguru virus (ULUV) (Kang et al., 2014)	Geata mouse shrews (*Myosorex geata*)	Tanzania
10. Makokou virus (MAKV) (Witkowski et al, 2016)	Noacks' round leaf bat *(Hipposideros ruber)*	Gabon

1.7 Disease in humans

Increased contact between humans and rodent excreta and especially when rodent numbers are high over an extended period of time directly affects transmission of hantaviruses to humans (Ramsden et al., 2009). The pathogenicity of certain hantaviruses such as Prospect Hill virus (PHV) is unknown suggesting there are hantaviruses that are non-pathogenic or asymptomatic in humans (Hardestam et al., 2009). In contrast, other hantaviruses cause significant disease such as SNV and ANDV with fatality rates of up to 50% (Hardestam et al., 2009). Transmission of hantaviruses through ingestion of contaminated

food has also been speculated (Hardestam et al., 2009). Humans are considered dead-end hosts in the transmission of hantaviruses which means that once the virus infects an individual it has no potential to be further transmitted with the exception ANDV which in rare cases is able to be transmitted from person-to-person (Hardestam et al., 2009).

1.8 Pathogenesis and Immunity

Hantaviruses are associated with two syndromes, renal insufficiency and acute respiratory disease. Both syndromes are associated with thrombocytopenia and vascular leakage caused by change in vascular permeability. Renal or pulmonary symptoms can range from mild to severe. A clinical incubation period ranging from one week to three weeks and sometimes lasting as long as six weeks has been reported. An early phase referred to as the prodome, occurs between 3-5 days after onset of illness with typical undifferentiated symptoms including fever, nausea, mylagia, malaise, backache, headache and abdominal pains sometimes accompanied by vision problems in the case of HFRS. Following the prodome phase (about 2-7 days later), organ failure manifest and cardiogenic shock and death may occur as a result of hypotension and lung edema. Positive prognosis of patients is indicated during the diurectic phase with rapid improvement of symptoms that may last for weeks (Kruger et al., 2011). The severity of the illness not only depends on the type of hantavirus but also host factors and it is often difficult to distinguish between phases (Kruger et al., 2011).

Neutralizing antibody responses are said to develop as early as onset of illness. Cross reactivity of neutralizing antibodies was observed in serum samples from acute-phase HFRS patients. Specific IgM and IgA are rapidly generated and IgG develops more slowly over time. The broad reactivity can hence be attributed to IgM during early phases of disease and conserved neutralizing epitopes (Kruger et al., 2011; Lindkvist et al., 2007).

Pathogenic and non-pathogenic hantaviruses share tissue tropism and replicate mainly in endothelial cells and macrophages (Maes et al., 2014). In patients with NE or HFRS, IgE titers are also elevated but their role is unclear. IgM and IgG responses to the NP are induced during the acute phase, while later during the course of illness, antibodies towards the Gn and Gc proteins are detectable (Prescott et al., 2005; Kruger et al., 2011). The fact that the glycoproteins are on the surface of the virion particle does not explain the earlier antibody response toward the NP but due to the abundance of this protein it is plausible to assume that there will be a earlier response against the NP. Early and efficient antibody

response may be useful in inhibiting dissemination of infected cells thereby the extent of damage caused by the virus. Protection from subsequent hantaviral infections has not been observed to determine if there is lasting humoral immunity, however re-stimulation due to viral persistence for example, of ANDV in convalescent patients may cause a rise in the antibody titers without re-infection (Kruger et al., 2011).

Cross-neutralization has been observed between hantaviruses. This can be explained by conserved B cell epitopes on the Gn and Gc proteins of different types of hantaviruses (Kruger et al., 2011). Cross neutralization was observed in laboratory infected hamsters and in rhesus monkeys after DNA vaccination. In addition, boosting with DNA vaccine increased cross-neutralization (Custer et al., 2003). Lifelong and highly differentiated memory T-cells have been detected in human survivors of ANDV, PUUV and HNTV (Kruger et al., 2011). Recent findings of a cellular and non-neutralizing immune response with ANDV and PUUV underline the importance of cellular immunity in hantaviral protection (Kruger et al., 2011).

Case fatalities of HCPS causing agents such as SNV and ANDV range from 30- 50%. These are the most prevalent hantaviruses in North and South America. Compared to the high fatality rates as a result of HCPS, other hantaviruses such as HNTV, DOBV, SEOV, PUUV and other HFRS causing agents, only account for up to about 15% fatalities of infected patients (Custer et al., 2003; Ramsden et al., 2009).

1.9 Diagnosis

Laboratory diagnosis is essential in differentiating between viruses causing a variety of similar symptoms. Clinical and epidemiological information is used in the diagnosis of hantavirus infection in humans but a definite diagnosis should not be based on clinical findings due to a milder or moderate disease outcome (Bi et al., 2008). Handling of hantavirus infected cells for the purpose of isolation and characterization requires level 3 or level 4 biosafety laboratory (BSL-3 or 4). Another disadvantage is that they are notoriously difficult to replicate in cell culture and are largely non-cytopathic. Viral RNA can be detected during the early stages of disease but this phase is short-lived. In cases where a virus cannot be isolated in cell culture, molecular assays such as real-time RT-PCR are employed on patient samples with an acute viral infection during the short viremic phase. In fatal cases, viral antigens are present in endothelial cells throughout the body and predominantly in the targeted organs such as the lung and kidney endothelial cells (Jonsson et al., 2010; Sjolander and Lundkvist., 1999).

Serological assays based on recombinant viral antigens have been employed for diagnosis of hantaviral infections by detection of specific antibody (IgM and IgG) response such as direct or indirect IFAs and enzyme-linked immunosorbent assays (ELISA), notably the IgM antibody-capture ELISA (Maes et al., 2004). Recombinant nucleocapsid proteins (rNP) have been produced in *E.coli* and other expression systems including mammalian and yeast systems and have been applied as antigens in ELISA (Maes et al., 2004; Sjölander et al., 1997; Yoshimatsu et al., 2014). Due to the fact that IgM rapidly decreases during the course of the disease and is less cross-reactive against heterologous hantaviruses when compared to IgG, it makes IgM detection a valuable component in rapid diagnosis of viral infections (Lundkvist et al., 1997).

Compared with other viral infections, there is high specificity of the IgG response toward the GNP with hantaviruses (Bharadwaj et al., 2000). Antigenic similarities are seen among hantaviruses. In an ELISA test, serum samples from patients with NE, which is caused by PUUV, were seen to cross-react with heterologous recombinant NPs against agents which included HNTV, DOBV, SEOV and SNV (Elgh et al., 1998). Increase in cross-reactivity was observed as the number of days progressed after onset of illness and most cross-reactivity was observed with the recombinant NP of SNV (Elgh et al., 1998). Although neutralization tests are the gold standard for differentiating between hantavirus types, they are not ideal for routine diagnosis. Hence, most serological assays for diagnosis are based on using recombinant or native antigens from heterologous hantaviruses.

1.10 Prevention and control

As yet there is no specific treatment available for hantavirus infections (Dargeviciute et al., 2002). In the year 2003, the annual human hospitalizations due to hantavirus infections were approximated to be 60,000 to 150,000 cases world-wide (Lee, 1996).

In 1990, an inactivated vaccine prepared in the brain of new born mice that protected the Korean military soldiers and the public from HFRS caused by HNTV was approved by WHO and put to use in the Republic of Korea. It required two doses and a third booster which was shown to play a role in its protective effectiveness (Cho and Howard, 1999; Park et al., 2004). Developing an inactivated virus vaccine requires the virus to be grown in bio-containment facilities. The virus should be chemically or physically inactivated and the addition of an adjuvant is often required for efficient performance. Alternatives to the killed virus vaccines have been investigated including the development of naked DNA vaccines

based on the M and S segment of hantaviruses in a mammalian expression vectors (Custer et al., 2003).

The vaccines expressed the Gn and Gc glycoproteins and induced neutralizing antibodies in experimental animal models including rodents and non-human primates. Protection induced by the vaccines was limited to SEOV, HNTV, and DOBV in hamsters (Custer et al., 2003). Passive protection of young rodents using neutralizing antibodies from infection can be seen however, the antibodies produced against the NP are not protective and they do not neutralize. In previous studies, it was seen that a plasmid construct using the M segment of SEOV was effective in eliciting neutralizing antibodies in mice, hamsters and new born rats (Hooper et al., 2001). Passive immunization of hamsters with either G1 and G2 neutralizing monoclonal antibodies protected the animals against HNTV infection (Schmaljohn et al., 1990) therefore substantiating the idea that protection can be attained by neutralizing antibodies alone (Hooper et al., 1999). The vaccine with the SEOV M segment was able to express the Gn and Gc glycoproteins eliciting a neutralizing effect in infected hamsters with three viruses causing HFRS. Higher neutralizing antibody titers were observed in rhesus monkeys which is said to be important in the development of a DNA vaccine that will protect human (Hooper et al., 1999; Hooper et al., 2001).

A vaccine comprising the full length M segment belonging to HNTV was also developed. The study tested the shared cross-reactivity of antibodies produced against HFRS agents in which they found that not only did the M gene segment of HNTV produce neutralizing antibody and protected against infection with HNTV but it also conferred protection against SEOV and DOBV but not PUUV meaning that neutralizing antibodies from the M gene of HNTV was able to protect from infection with the two other HFRS agents. Dual vaccination of non-human primates has been performed using a DNA vaccine incorporating both the M gene segments of HNTV and ANDV for protection against both HFRS and HCPS and the animals were shown to produce cross neutralizing antibodies that were long lasting (Hooper et al., 2006) A phase one clinical study has been carried out in which a naked DNA vaccine comprising of the M gene segments of two HFRS causing agents was administered by Intramuscular electroporation (I-EP) (Hooper et al., 2014).

The first rule of preventing infection with hantavirus by means of physical methods is to avoid rodents and exposure to their excreta. For people living in rural or rodent infested areas it is noteworthy to know that although these rodents prefer living outside in the fields,

they will from time to time get inside the houses for food, water and cover and therefore it is important to take the necessary protective measures if you live in such an area. These precautionary measures include rodent-proofing homes by removing all trash or junk, covering food, and airing cabins that are kept closed most times as well as removing trapped rodents and their dropping and disinfecting the areas where they are trapped (Schmaljohn and Nichol., 2007).

1.11 Recombinant technology

Recombinant DNA technology facilitates expression of target genes. Genetic material of an organism can be modified to obtain desired characteristics and products. DNA encoding proteins of interest can be inserted into a DNA vehicle known as a plasmid vector. Vaccine development and pharmaceutical products are some of the applications of recombinant DNA technology (Lomedico., 1982). In the current growing research field, new devices, products and approaches are being developed through recombinant DNA technology and are applied in agriculture, health and environment for example, recombinant insulin used to treat diabetics (Lomedico., 1982; Gualandi-Signorini and Giorgi, 2001).

Serological assays have proven to be suitable for surveillance of the immune response in humans and animals and are frequently used as a tool for investigating evidence for the presence of new or emerging pathogens. Molecular techniques such as PCR are rapid and also have application in identification of virus in tissue (Warnes et al., 1994). However molecular assays depend on samples that are viremic whereas serological assays can be used to screen samples for evidence of past infection. Traditionally, ELISAs and IFAs dependent on the use of crude antigens from whole virus or infected cells that requires a BSL 3 or 4 laboratory . With recombinant DNA technology, a wide range of proteins that were previously unavailable to use because of biosafety issues or were difficult to prepare in native form and problems with stability, could now be used. Recombinant proteins have therefore been employed as key tools in diagnostic assays (Warnes et al., 1994). Recombinant proteins were initially produced primarily from *E.coli* cells and other prokaryotic hosts but there can be problems with sensitivity and background interference (Warnes et al., 1995). Syphilis recombinant proteins were the first to be developed and put into commercial assays (Young et al., 1998). Whole virus was the basis of hantavirus discovery using IFA with fixed organs or cultured viruses that were reacted with positive sera. With the development of recombinant technology, the use of recombinant antigens,

mostly the NP of hantaviruses, have become the basis of a safe diagnostic tool in serology and commercial products containing a mixture of recombinant NP of hantaviruses from Europe, Asia and America are available for purchase (Lee et al., 1978; Klempa et al., 2006, 2010).

1.12 Problem identification

Hantaviruses are the causative agents of zoonotic diseases. They are considered emerging pathogens that are distributed throughout the world. They were initially thought to exist in Europe, Asia and America. More recently, hantaviruses have been identified in Africa however, there is limited data on African hantaviruses and its seroprevalence. Hantavirus infections are a cause for public concern as they are the causative agent of up to an estimated cases of 150,000 annually. They are transmitted by aerosolized excreta of reservoir hosts and they are the cause of HFRS or HCPS in humans. They were thought to be primarily transmitted by rodent reservoirs but the recent discovery of indigenous African hantaviruses since 2006 has led to the identification of novel reservoirs such as shrews and bats.

There are limited seroprevalence studies performed in Africa to detect IgG in humans. The emergence of hantaviruses could have significant public health implications. Currently there are commercial assays available in the form of ELISA with antigens specifically for detection of European and American hantaviruses. In a previous study performed in our Department, 176 sera from patients with acute febrile illness and suspected tick-bite fever were screened using these commercial assays. Using the Eurasia assay, 17/176 (10%) of samples were positive, whereas 11/176 (6%) reacted with the American-based antigens (Mathengtheng L, PhD thesis unpublished 2015). An additional 11 samples were regarded as borderline for the Eurasia assay. A total of eight samples had detectable IgG antibody against hantavirus using both assays. These results indicate that further serological studies are warranted. Although there is some serological cross-reactivity between hantaviruses, there are antigenic differences. Hence it was considered that the preparation of a recombinant African hantavirus antigen based on SANGV could have application in serological surveillance studies. In this study the preparation and characterization of an African hantavirus recombinant antigen and its application in serological assays compared with European and American NPs and commercial assays was investigated.

1.13 Aim and objectives

Aim

The aim of the study was to develop serological assays using recombinant Sangassou NP antigen for the detection of antibody against African hantaviruses

Objectives

1. The preparation of a plasmid construct for expression of recombinant Sangassou NP antigen in mammalian cells and characterization of the expressed recombinant antigen.

2. Development of an immunofluorescent assay (IFA) using transiently transfected cells.

3. Application of IFA slides for screening human sera for IgG antibody against hantaviruses and comparison of commercial ELISA kits with an in-house assay.

Chapter 2: Preparation and characterisation of the recombinant nucleocapsid protein of Sangassou virus

2.1 Introduction

Serological assays are the basis of laboratory diagnosis of human hantavirus infections. The classic approach involves performing an indirect IFA or ELISA for detection of immunoglobulin class G (IgG) and M (IgM) antibodies (Schlegel et al., 2012). Virus isolation is the most reliable method of accurately identifying an infecting hantavirus when coupled with PRNT. These methods are however very laborious and some hantaviruses are known to be difficult to isolate in cell culture (Chu et al., 1994; Schmaljohn et al., 1983). Due to the early and long-lasting immune response induced by the NP of hantaviruses, the NP antigen is frequently used for the detection of IgG antibody responses. The NP has a molecular weight of approximately 50 kilo Daltons (kDa) and other roles for the protein, in addition to protecting and encapsidating the viral genome, have been proposed (Hussein et al., 2011).

Molecular assays such as RT-PCR are quick and easy to use for detection of the viral nucleic acid and play an important role in the identification of hantavirus in rodent reservoirs because of the chronic infection that is established in rodents. However, in human disease nucleic acid amplification tests are seldom used as the period of viremia is shortlived and therefore serological assays play an important role in surveillance or for determination of the immune status of human populations. Traditionally, serological assays have been developed using native antigens that require culturing the virus. However, hantaviruses require high biocontainment to culture which limits the number of laboratories that can handle the virus. Recombinant technology provides a safe and effective means of preparing viral antigens for development of serological assays such as IFA and ELISA without the need for biocontainment (Yoshimatsu et al., 1993; Kruger et al., 2011; Fooks et al., 1993). Other studies have used the recombinant NP of Crimean Congo hemorrhagic fever virus as a specific, sensitive, safer and cost effective tool to detect IgG antibodies in human serum samples and a correlation with commercially available diagnostic tools was observed. Protein expression in insect, bacteria and plant systems have been explored and their application in an indirect ELISA show high specificity and sensitivity. This is especially important for surveillance and rapid diagnosis where high biosafety laboratories would otherwise be needed for live virus production (Atkinson et al., 2016; Zhou et al., 2011; Samudzi et al., 2012; Beasley et al., 2004; Warnes et al., 1994)

Recombinant DNA technology involves the manipulation of DNA for antigen production. Recombinant antigens have the advantage of reducing cost of production and serving as a safe tool in diagnostic applications and for vaccine development (Fox and Klaas, 1989). It also gives the option to modify the gene for enhanced expression of the gene and incorporating tags to facilitate purification of the protein (Fox and Klaas, 1989).

Recombinant hantavirus antigens have been prepared in insect, yeast and bacterial cultures (Yoshimatsu et al., 1993; Luan et al., 2006; Kallio-Kokko et al., 2000; Kruger et al., 2011). Bacterial expression systems have drawbacks in that proteins lack post-translational modifications such as phophorylation and glycosylation as well as correct folding of the proteins compared with mammalian expression systems, which allow for post translational modifications, correct folding and protein solubility (Fox and Klaas, 1989).

Commercial assays are available for detecting IgG antibody against hantaviruses in humans using antigens representing either European or American strains of the virus. However, more recently an African hantavirus has been identified and in the absence of convincing serological surveillance data from African populations for the presence of hantaviruses in southern Africa, it was considered appropriate to prepare an African hantavirus antigen for use as a serological tool. The aim of this chapter was to prepare and characterize the recombinant NP of an African hantavirus, SANGV. It was chosen because to date SANGV is the only African hantavirus that has been isolated in cell culture and fully characterized. Hence, sequence data for the gene encoding the NP was available (Klempa et al., 2010; 2012). Commercial assays for diagnosis and surveillance usually employ recombinant antigens from Europe, Asia such as the HNTV, PUUV and DOBV or from America such as SNV and ANDV. The extent of serological cross reactivity between African hantaviruses and American and European has not been investigated and hence it is useful to compare for future application in surveillance studies. A suitable mammalian expression vector and host cell were selected for expression of the protein. Characterisation of the expressed protein was performed to confirm expression of the desired protein.

2.2 Methods and Materials

2.2.1 Preparation of construct for expression of recombinant SANGV nucleocapsid antigen

The complete nucleotide sequence of the ORF of SANGV strain SA14 S gene, accession number JQ082300, was retrieved from the online sequence database GenBank (*http://www.ncbi.nlm.nih.gov/genbank/*). The complete S gene is comprised of 1746 nucleotides with an ORF of 1290 base pairs that encodes the NP of SANGV. The ORF was codon optimized (http://www.jcat.de/) for expression in BHK-21 cells using the rare codon analysis tool provided by GenScript (*http://www.genscript.com/*).

The codon optimized gene was synthesized by GenScript (GenScript, Piscataway, USA) and supplied in the mammalian expression vector pcDNA 3.1(+). The construct was designated SANGV_pcDNA3.1(+). A Kozak sequence at the 5' N-terminus of the gene was included for initiation of translation, and a C-terminus HisX6 tag was included for purification and detection of the recombinant protein. In addition, the gene was modified at the 5' and 3' ends to include restriction sites for EcoRI and Xbal enzymes respectively, to facilitate characterization of the construct.

The expression vector, pcDNA 3.1(+) is comprised of 5.4 kilobase pairs (kbp) and contains, amongst other important elements, resistance genes for neomycin/kanamycin (Appendix A). These resistance genes facilitate selection of a stable cell line for constitutive protein expression. The human cytomegalovirus (CMV) immediate-early promoter is also present and facilitates elevated levels of expression in a range of mammalian cells and the multiple cloning site, comprised of restriction sites is used for insertion of foreign genes into the vector.

The synthesized gene cloned into pcDNA3.1 (+) was supplied in lyophilized form and reconstituted in NFW. For long-term storage, the DNA was frozen at -20 ^{o}C. A stock of plasmid DNA was prepared by transforming chemically competent *Escherichia coli* (*E. coli*) cells, Invitrogen™ OneShot®TOP10 chemically competent *E. coli* cells (Invitrogen, Carlsbad, Ca), with a transfection efficiency of ≥ 1 × 10^{9} cfu/µg. The competent cells were transformed using the heat shock method according to manufacturers' instructions. Briefly, agar plates were prepared using Luria Bertani (LB) broth (Appendix B) containing 100µg/ml ampicillin (amp) and were preheated to 37^{o}C in the incubator and the cells were thawed on ice. Amp

was used due to the presence of the *bla* gene in pcDNA3.1 (+) that confers resistance to amp allowing only cells that are positively transformed to grow on the selective agar plates. A 1µl aliquot of SANGV_pcDNA3.1(+) DNA construct (concentration 500-700ng/µl) was added to 50µl of competent cells. The cells and DNA mixture were heat shocked at 42 ^{o}C for 50 seconds. Super Optimal broth with Catabolite repression (SOC) media (Appendix B) was added to facilitate growth and cells were incubated for 90 minutes at 37 ^{o}C with shaking. The transformation mix was plated on plates and incubated at 37 ^{o}C overnight (O/N). The following day three colonies were selected and tested for positive transformants. The control plasmid DNA, pUC19, was used to confirm the transformation efficiency. To test for positive transformants, colonies were selected and inoculated into 5ml LB broth containing 100µg/ml amp. Bacterial cultures were incubated O/N at 37 ^{o}C with shaking at 200 rpm.

2.2.1.2 Plasmid extraction

Plasmid DNA was purified from overnight cultures using the PureYield™ Plasmid miniprep system (Promega, Winsconsin, USA) according to manufacturers' instructions. Briefly, 3ml bacterial culture was processed. Unless otherwise stated, all centrifugation steps were performed at 16000 x g. Cells were harvested by centrifuging and resuspending in distilled water. The cells were lysed using lysis buffer supplied in the kit followed by addition of neutralization solution (stored at 4^{o}C). The lysed cells were centrifuged to allow for gradient fractionation and the supernatant containing plasmid DNA was processed. Endotoxin removal wash was added to the column and the DNA was eluted in 30µl of NFW and stored at 4^{o}C. The DNA concentration was determined using the Nanodrop™ 2000 (Thermo Fisher Scientific, Wilmington, USA). Glycerol stocks for each colony were prepared by addition of glycerol to the remaining 2ml of the O/N bacterial culture of transformed cells to a final concentration of 15% and were frozen at -80^{o}C for long-term storage.

2.2.1.3 PCR

To confirm the presence of the SANGV NP gene encoding the recombinant NP in the plasmid construct, a PCR was performed using purified plasmid DNA as template. The PCR was performed using the Promega GoTaq® DNA Polymerase kit (Promega, Madison, USA) according to manufacturers' recommendations and preparation of a PCR mix as shown in Table 2.1. The S gene encoding the NP of SANGV was amplified using T7 and BGH primers (IDT, Coralville, USA) (Table 2.2).

Table 2.1: Reaction mixture composition including final concentrations used

Reagent	Final volume	Final concentration
$MgCl_2$	2µl	1,0mM
5X Green GoTaq® Flexi buffer	10µl	1X
PCR nucleotide mix	1µl	0,2mM
Forward primer	1µl	20pmol/µl
Reverse primer	1µl	20pmol/µl
GoTaq® Hot start polymerase	0,25µl	1,25U
Template DNA	1µl	29,7ng/µl
Nuclease free water	33,75µl	
Total reaction volume	50µl	

Table 2.2: Primer sequences used in PCR for amplification of the S gene encoding the NP of SANGV in pcDNA 3.1(+).

Primer:	Sequence:	Length (nt):	Melting temperature (Tm):
T7	5'- TAA TAC GAC TCA CTA TAG GG - 3'	20	56^{o}C
BGH	5'- TAG AAG GCA CAG TCG AGG – 3'	18	61^{o}C

The following cycling conditions were used for the PCR performed on a GeneAmp 9700 (Applied Biosystems, Life Technologies, Oregan, USA): an initial denaturation at 95^{o}C for 2 minutes, followed by 25 cycles of denaturation at 94^{o}C for 30 seconds, annealing of primers at 53^{o}C for 30 seconds and extension at 72^{o}C for 1 minute. A final extension step was performed at 72^{o}C for 5 minutes and thereafter the reaction was held at 4^{o}C.

PCR products were separated and visualized by electrophoresis. A 6 x loading dye (Fermentas, Illinois, USA) was used to track migration of PCR amplicons on a 1% agarose gel (Appendix B). Ethidium bromide at a concentration of 0,6 µg/ml was used for visualization of PCR products. The O'Gene Ruler DNA ladder mix #SM1173, (Fermentas, Illinios, USA) with DNA fragments of 100 bp to 10, 000 bp, was used to predict the size of the amplified products. Separation was performed for 35 minutes using a Bio-Rad PowerPac Basic system

(Bio-Rad, California, USA) at 80 volts (V). Bands were observed using a UVpro transilluminator (UVItec, Cambridge, UK).

The amplified S gene was purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Wisconsin, USA) according to manufacturers' instructions. Briefly, membrane wash solution was added to the amplicon. DNA separation was achieved by adsorption to silica membrane present in the SV minicolumn and an aliquot of wash solution was added. DNA was eluted in NFW and the DNA concentration was determined using the Nanodrop™2000. PCR clean up of amplicon was done in order to remove unincorporated dNTPs, primers and salts from the PCR reaction. Purified DNA was used for confirmation of sequence.

2.2.2 Confirmation of SANGV S gene construct for expression

The nucleotide sequence of the gene encoding the NP of SANGV was determined using Sanger sequencing. Sequencing was performed using the BigDye® Terminator V3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems, London, England). The plasmid, pGEM-32f(+) and M13 primer supplied in the kit and were used as a control for the sequencing reaction. Sequencing reactions were performed using the GeneAmp®PCR system 9700 under the following conditions: initial denaturation was carried out at 96^{o}C for 1 minute, and 30 cycles of denaturation at 96^{o}C for 10 seconds, primer annealing at 50^{o}C for 5 seconds and extension at 60^{o}C for 4 minutes, and finally the tubes were held at 4^{o}C.

Table 2.3: Reaction mix for bidirectional sequencing of SANGV S gene using T7 and BGH primers.

Reaction components:	**Reaction volume:**
Ready reaction	1 µl
Sequencing primer (T7 or BGH)	4 µl(0.8pmol/µl)
Dilution buffer	2 µl
Template DNA	1 µl(10-40ng)
Nuclease- free water	2 µl
Total volume	10 µl

Post-reaction clean up was performed by diluting 0,5 M EDTA (pH8, 0) to 125 mM EDTA with distilled water. The sequencing reaction volume was adjusted to 20 µl by adding 10 µl

of NFW, and a 5 µl aliquot of 125 mM EDTA and 60 µl of absolute ethanol was added. The sequencing reaction mixture was vortexed for 5 seconds and allowed to precipitate at room temperature (RT) for 15 minutes. After centrifugation at 4°C for 20 minutes, the supernatant was aspirated and caution was taken not to disturb the DNA pellet. Thereafter, a 500µl aliquot of 70% ethanol was added to wash the pellet by centrifugation at 14 000 x g at 4°C for 10 minutes. Residual ethanol was allowed to evaporate at 37°C for 2 hours and samples were stored in the dark at 4°C and submitted for electrophoresis at the Department of Microbial, Biochemical and Food Biotechnology at the University of the Free State. Sequencing data was analysed with ChromasPro version 1.6 (Technelysium, South Brisbane, Australia).

2.2.3 Control plasmid expressing the green fluorescing protein (GFP)

A pcDNA3.1(+) construct expressing green fluorescing protein (GFP) (prepared in a related study by N Viljoen) was used as a positive control for transfection experiments.

2.2.4 Transfection of mammalian cells

BHK-21 cells (American Type Culture Collection (ATCC) number CCL-10) were maintained in T25 cell culture flasks (Corning, New York, USA) and allowed to reach 70-90 % confluency before they were passaged. The cells were grown in Eagles Minimum Essential Medium (EMEM) (Lonza, Verviers, Belgium) supplemented with 2mM L-glutamine (Life Technologies, New York , USA), 5% fetal bovine serum (FBS) (BiochromAG, Berlin, Germany) , 100 units per ml (U/ml) penicillin and streptomycin (pen/strep) (Lonza, Verviers, Belgium) and 1% non-essential amino acids (NEAA's) (Lonza, Vervier, Belgium) growth medium. The cells were washed with 2x2ml phosphate buffered saline (PBS) (Lonza, Verviers, Belgium) and dissociated using 250µl of 10X trypsin (Lonza, Verviers, Belgium) after. Cells were resuspended in 3ml growth medium and seeded according to the appropriate ratio depending on when the cells were needed and incubated at 37°C in 5% CO_2 .

Cells were counted using a Neubauer haemocytometer under an inverted microscope. To obtain 70-90% confluency for transfection, cells were seeded at 1×10^5 cells / well in 24-well plates in a total volume of 1 ml of media. The cells were grown on coverslips for downstream processing. Cells were transfected using Lipofectamine™ 3000 transfection reagent (1mg/ml) (Life Technologies, Ca, USA) according to manufacturers' instructions. Briefly, Lipofectamine™ 3000 was diluted in 50 µl of OptiMEM® I reduced serum medium (Gibco, Presley, UK). The SANGV_pcDNA 3.1(+) construct was diluted in 50µl Opti-MEM®I

medium and incubated at RT. A range of DNA concentrations: 0,5µg, 2,0µg and 3,5µg were used and 0,75µL and 1,5µL of Lipofectamine ™ 3000 reagent with P3000 reagent from 1 to 3,5µL were allowed to form a complex by mixing and incubating at RT for 15 minutes. Growth media in the 24 well plates was replaced with growth media without antibiotics. The DNA lipofectamine complexes were added to each well where cells were grown on coverslips. The same transfection conditions were used with pcDNA3.1(+) expressing GFP. Cells were incubated for 18 hours in a humid environment for favorable conditions of 37^{o}C in a CO_2 incubator for cell growth.

Indirect IFA using mouse monoclonal antihistidine antibody (Roche, Indianapolis, USA) was performed for initial confirmation of the expressed recombinant NP of SANGV. Transiently transfected cells were fixed on the coverslips that were used for cell attachment in 24 well plates, by addition of a cold solution of 1:1 methanol (Merck, Darmstadt, Germany) and acetone (Merck, Darmstadt, Germany) for 20 minutes at -20°C. Excess blocking solution (Appendix B) was applied and the cells incubated for 20 minutes at RT to prevent scavenge proteins from binding to the empty spaces to lower the background. Primary antibody in the form of mouse monoclonal antihistidine antibody (Roche, Indianapolis, USA) diluted 1:100 in blocking buffer was added to the cells and incubated for 90 minutes at 37°C in a humidified container. The cells were washed with 1% Tween® 20 (Promega, Wisconsin, USA) in PBS and reacted with a secondary antibody, antimouse IgG conjugated to fluorescein isothiocyanate (FITC) (KPL, Maryland, USA), diluted 1:20 in 0.1% of Evans blue solution (Merck, Darmstadt, Germany) in PBS. Slides were incubated for 30 minutes at 37°C in a humid container and unbound antibody was removed by washing with 1% Tween® 20 in PBS. Similarly, cells expressing the GFP were fixed with cold 1:1 acetone:methanol solution at -20 °C. Cells were dried and mounted on microscope slides for visualization under the OPTIPHOT fluorescence microscope (Nikon, Tokyo, Japan). Slides were stored at 4°C for further analysis.

2.2.5 Purification and quantification of recombinant NP of SANGV produced in BHK-21 cells

For analysis of expressed protein, the cells were incubated for 24 hours in 24 well culture plates and harvested by washing with cold PBS and directly applying NET/BSA. Cells were incubated on ice for 30 minutes with shaking. The cell suspension was centrifuged at maximum speed in a benchtop microcentrifuge and the supernatant frozen at -20^{o}C until

use. The recombinant SANGV NP was purified from the cell extract using the Protino® Ni-TED 1000 columns (Macherey-Nagel, Düren, Germany) according to the manufacturers' instructions. Protino®NiTED products enable fast and convenient purification of recombinant polyhistidine tagged proteins by immobilized metal ion affinity chromatography (IMAC). Protino®Ni-TED is a dry silica-based resin precharged with Ni2+ ions. Protein binding is based on the interaction between the polyhistidine tag of the recombinant protein and immobilized Ni2+ ions. The chelating group of Protino® Ni-TED is based on TED (tris-carboxymethyl ethylene diamine), a strong pentadentate metal chelator. TED occupies five of the six binding sites in the coordination sphere of the Ni2+ ion. Compared to TED, other chelating groups such as NTA (nitrilotriacetic acid) have four binding sites available for the Ni2+ ion. The additional chelation site of TED with Ni2+ minimizes metal leaching during purification and increases specificity for polyhistidine-tagged proteins. As a result, target protein of excellent purity is eluted from the column.

Briefly, the Protino® Ni-TED 1000 Packed Column was equilibrated by applying lysis-equilibration-wash (LEW) buffer and the cell extract was added to allow binding of the proteins to the column. The column was washed twice with LEW buffer and subsequently protein was eluted in four 500 µl fractions using 1x elution buffer containing imidazole.

2.2.6 Characterization of recombinant NP of SANGV using SDS-PAGE

Using the method described in section 2.2.4, BHK-21 cells grown in 6-well cell culture plates (Corning, New York, USA) were transfected using SANGV_pcDNA3.1(+) using Lipofectamine® 3000 reagent. BHK-21 cells were seeded at 5 ×10 ^{5} cells/cm^{2} and allowed to reach 70-90% confluency before transfection. A 6.75µL aliquot of Lipofectamine®3000 reagent was diluted in 250µl of OptiMEM ®I reduced serum medium. An aliquot of 13.5 µl lipofectamine reagent and 9,0 µg of SANGV_pcDNA 3.1 (+) DNA were diluted in 250 µl of OptiMEM® I reduced serum medium. The diluted Lipofectamine and DNA were combined and incubated at RT for 15 minutes to allow for Lipofectamine/ DNA complexes to form. The cells were incubated in 5% CO_2 incubator at 37^{o}C for 18-24 hours to allow for sufficient protein expression.

Analysis of protein was done using SDS-PAGE where a 12% resolving gel was prepared as illustrated in Table 2.4 and poured into pre-assembled electrophoresis apparatus (BIO-RAD, USA). Before polymerization, a top layer of amyl alcohol was applied.

Table 2.4: Preparation of a 12 % resolving gel for protein analysis using SDS-PAGE.

Reaction component:	Reaction volume:	Final concentration:
1.5M Tris-HCl (pH8,8)	2,75 ml	0,4125M
30 % Bis-acrylamide	4 ml	12%
10 % SDS	0,100 ml	0.1%
50% Glycerol	2 ml	10%
dH_2O	0,975 ml	-
10 % APS (freshly prepared)	0,150 ml	0.15%
TEMED (add last)	0,025 µl	0,25%
Final volume	10 ml	

Upon polymerization of the resolving gel, the amyl alcohol was removed and a 4% stacking gel, prepared as shown in Table 2.5, was applied to the apparatus and a comb was inserted before polymerization of the gel.

The purified protein samples were diluted in 5 x Lane Marker Reducing Sample Buffer (ThermoScientific, Illinois, USA) and samples were incubated for 5 minutes at 95^{o}C . The samples were separated at 60 V for 15 minutes and 140 V for 40 minutes in 1× Tris-Glycine-SDS electrophoresis buffer (25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3) (Fermentas, USA). The PageRuler™ prestained protein ladder (Thermo Scientific Illinois, USA) was used for the estimation of proteins in the 10-170kDa range. The gel was stained in approximately 200ml of staining solution containing 45% methanol, 10% glacial acetic acid and 0,2% Coomasie Brilliant Blue (Fluka, Buchs, Germany) with gentle shaking O/N for visualization of the proteins and destained using destain solution (Appendix B)

Table 2.5: Preparation of a 4% stacking gel for protein electrophoresis of the purified NP of SANGV

Reaction component:	Reaction volume:	Final concentration:
1,5M Tris-HCl (pH 6,8)	1,375 ml	0,412M
30% Bis-acrylamide	0,667 ml	4%
10% SDS	0,050 ml	0,1%
50% Glycerol	1 ml	2.5%
dH_2O	1,808ml	-
10% APS(freshly prepared)	0,075 µl	0,075%

TEMED (add last)	25 µl	0.25%
Final volume	5 ml	

2.2.7 Western blot analysis of the recombinant NP of SANGV using anti-His antibodies

Characterization of the C-terminal his6 tagged NP of SANGV was performed by Western blot analysis using the Pierce®Fast Western Blotting Kit (Thermo Scientific, Illinois, USA). The SDS-PAGE gel was incubated in transfer buffer for 15 minutes with gentle agitation. The membrane was activated by soaking in methanol for 5 minutes and thereafter rinsed with deionised water for 2 minutes while gently shaking and was soaked in transfer buffer (Appendix B) for 3 minutes. A Biotrace™ polyvinylidene fluoride (PVDF) transfer membrane (PALL Corporation, Florida, Mexico) with a pore size of 0,2µm was activated in methanol for 5 minutes with gentle agitation and then the protein transferred.

Subsequently, proteins were transferred using a Trans-Blot® SD semi-dry electrophoretic transfer cell (Bio-rad, California, USA) at 20V for 60 minutes after which the PVDF membrane was washed twice with Tris-buffered saline (TBS) at a pH of 7.5 (Appendix B) for 10 minutes to remove any residual methanol. The membrane was incubated at 4°C O/N with mouse monoclonal antihistidine antibody diluted at 1:200 in antibody diluent solution provided in the kit. Unbound antibody was removed by washing the PVDF membrane three times using TBS containing 0,05% Tween® 20 for 10 minutes with gentle agitation. The PVDF membrane was incubated in optimised horseradish peroxidase reagent diluted at 1:10 in antibody diluent for 15 minutes with gentle agitation.

The membrane was washed using TBS containing 0,05% Tween® 20 (Appendix B) three times for 10 minutes with gentle agitation to remove any unbound antibody and reduce background, and a final wash of 10 minutes with gentle agitation using TBS was performed. The SuperSignal® West Pico Substrate was prepared by a 1:1 dilution of SuperSignal® West Pico Luminol/Enhancer solution and SuperSignal® West Stable Peroxide solution. The PVDF membrane was exposed to SuperSignal® West Pico Substrate for 5 minutes with gentle agitation, covered in clear plastic wrap to prevent drying and bound antibody visualised by exposure of the membrane and CL-XPosure™ X-ray film (Thermo Scientific, Illinois, USA) to X-ray for 30 seconds. The image was developed for 3 minutes in developer solution (Axim,

Gauteng, RSA) and fixed for 2 minutes in fixer solution (Axim, Gauteng, RSA). The film was rinsed with water to remove any residual fixer solution.

2.3. Results

2.3.1 Preparation of SANGV_pcDNA3.1(+) construct

The ORF of SANGV S gene was codon optimized for expression in mammalian cells. BHK cells were used for expression and the codon was optimized in murine cells. For maximal expression of the protein in BHK-21 cells, several factors regulating and influencing high levels of protein expression such as mRNA structures, poly A site, RNA instability and GC content were taken into consideration using the Optimum Gene algorithm. Before optimization, the native gene of SANGV NP comprised of tandem rare codons that could negatively affect translation of the protein. The distribution of codon frequency usage demonstrated by the CAI value of 0.67 is indicative of infrequent recognition of several codons of the SANGV gene by mammalian cells (Figure 2.1). The CAI index is an effective measure in the optimization process used to measure codon bias in predicting gene expression levels and similarities between codon usage of a gene and codon usage of the host. Ideally, for high level expression of the protein the CAI should be 1.00 with a value of 0.8 still considered good for protein expression in desired expression organisms (Figure 2.2).

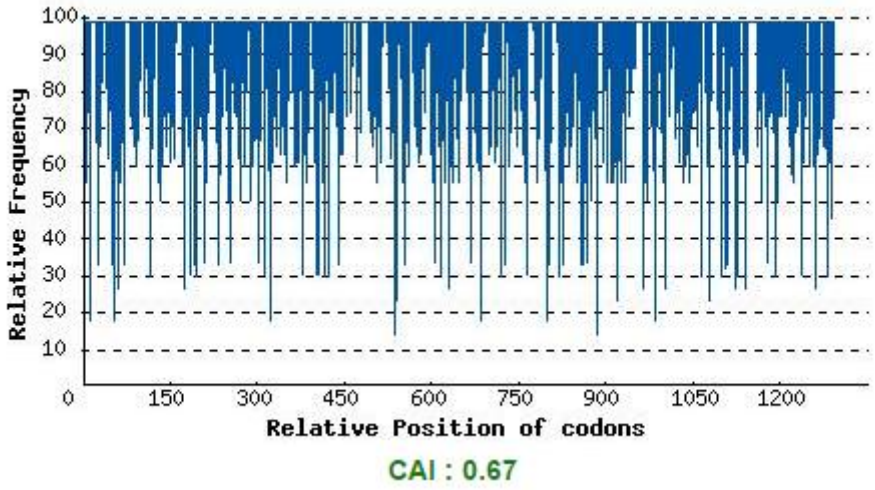

Figure 2.1: Distribution of codon usage frequency along the length of the ORF of the S segment of SANGV strain SA14 before optimization

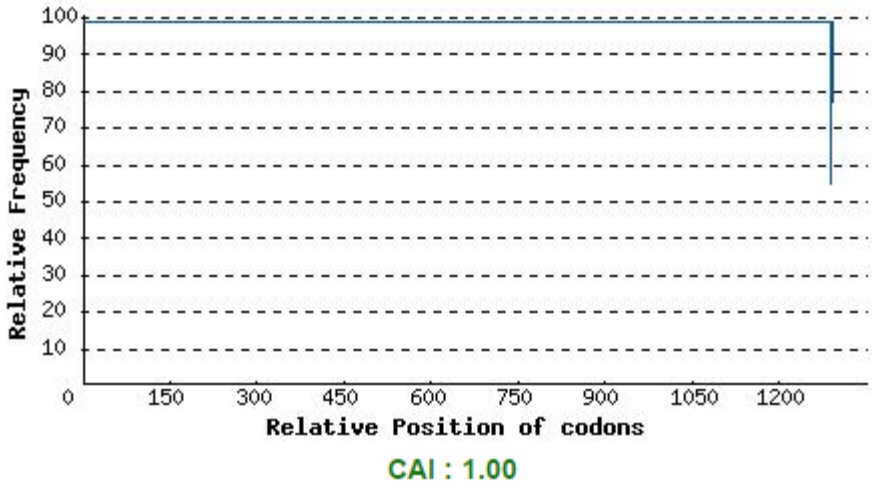

Figure 2.2: The distribution of codon usage frequency along the length of the ORF of the S gene of SANGV after optimization

Figure 2.3 & 2.4 illustrated further the recognition by the targeted cells shown as the percent distribution of less frequently utilised codons from SANGV NP gene. Grouping of codons according to the usage by rat cells during the optimization process show 11-20% as the group less frequently used and 91-100% being the group that is most frequently used by the target organism. Only 34% of the codons for protein expression were frequently used by the target cells however, this was increased to 99% after codon optimization.

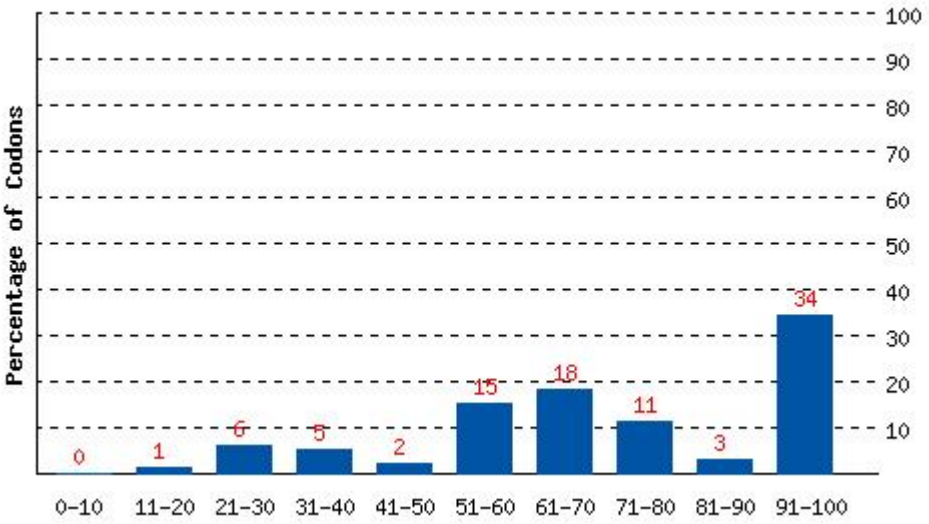

Figure 2.3: Percent distribution of codons in computed codon quality groups before optimization

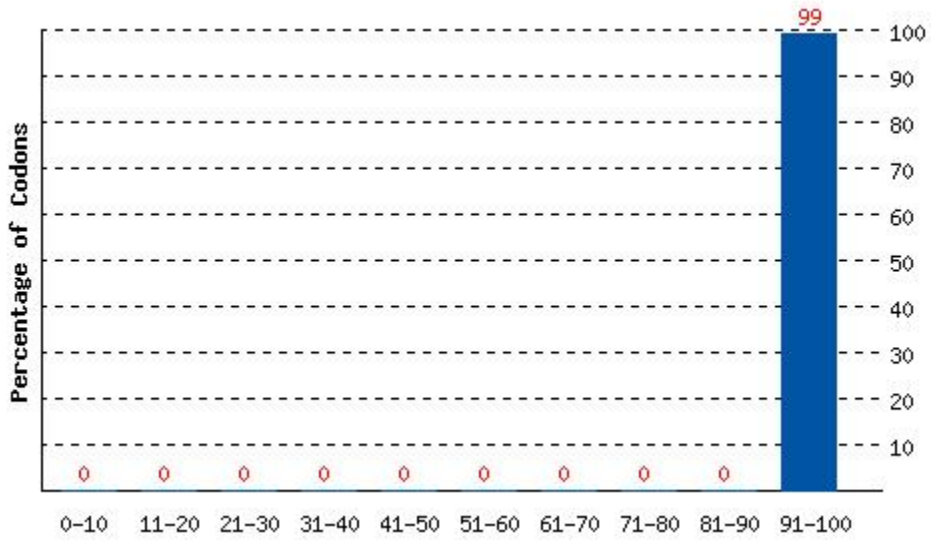

Figure 2.4: Percent distribution of codons in computed codon quality groups after optimization

A GC content of between 30 - 70% is ideal, unfavourable peaks are said to have adverse effects on the efficiency of transcription and translation, and therefore optimization was necessary to stabilize the mRNA. Before optimization, the average GC content of 41.99 was shown in Figure 2.5 and increased to 63.77 after optimization of the NP gene of SANGV in Figure 2.6.

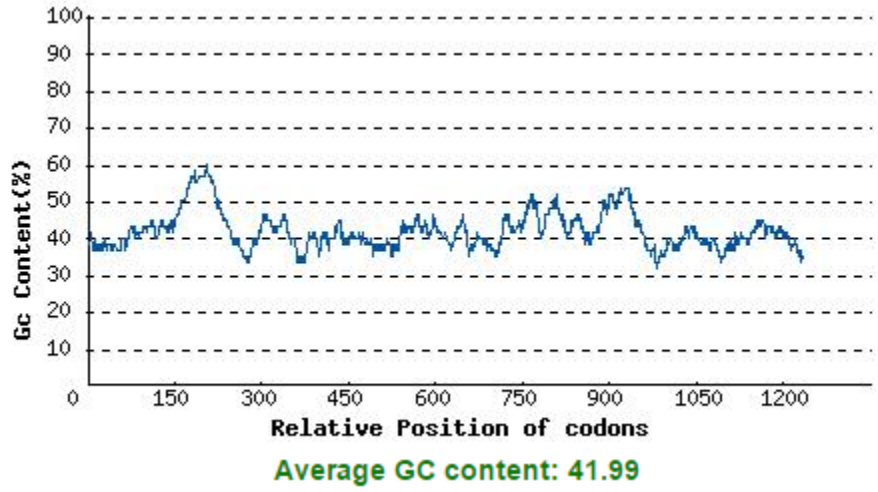

Figure 2.5: Average GC content before optimization

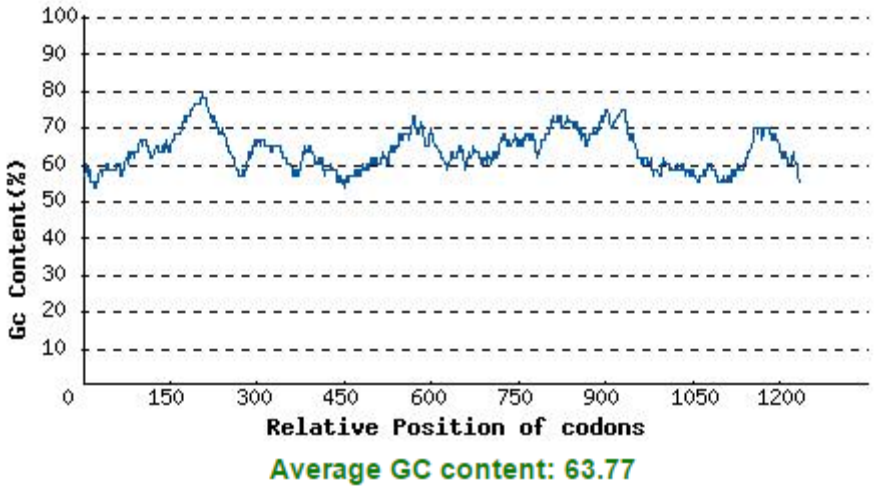

Figure 2.6: Average GC content after optimization

The nucleotide and amino acid sequences of the codon optimized recombinant NP gene of SANGV and native S gene of SANGV SA14 are aligned as shown in Figure 2.7. The codon optimized NP shares amino acid sequence with the native SANGV SA14 NP and this indicates that the expressed protein will be that of SANGV.

In Figure 2.7 the native SA14 S gene and recombinant S gene encoding the NP of Sangassou were translated using an online tool, ExPASy (web.expasy.org/translate/) and aligned using Clustal Omega tool (www.ebi.ac.uk/Tools/MSA/Clustal). A 100% identity of the recombinant gene to the native was observed**.**

Figure 2.7: Amino acid sequence alignment of the native S gene of SANGV and recombinant NP gene after translation. The sequence of predicted protein based on nucleotide from sequence of codon optimised gene and native gene (SA14) shows a 100% identity using Clustal Omega tool. SR in the codon optimized gene is the XbaI site added on the 3'end as a modification.

PCR was performed after transformation of competent *E.coli* cells for plasmid extraction using the T7 and BGH reverse primers to amplify the S gene which is 1393bp and can be seen in Figure 2.9 with the molecular weight marker, negative control and S gene.

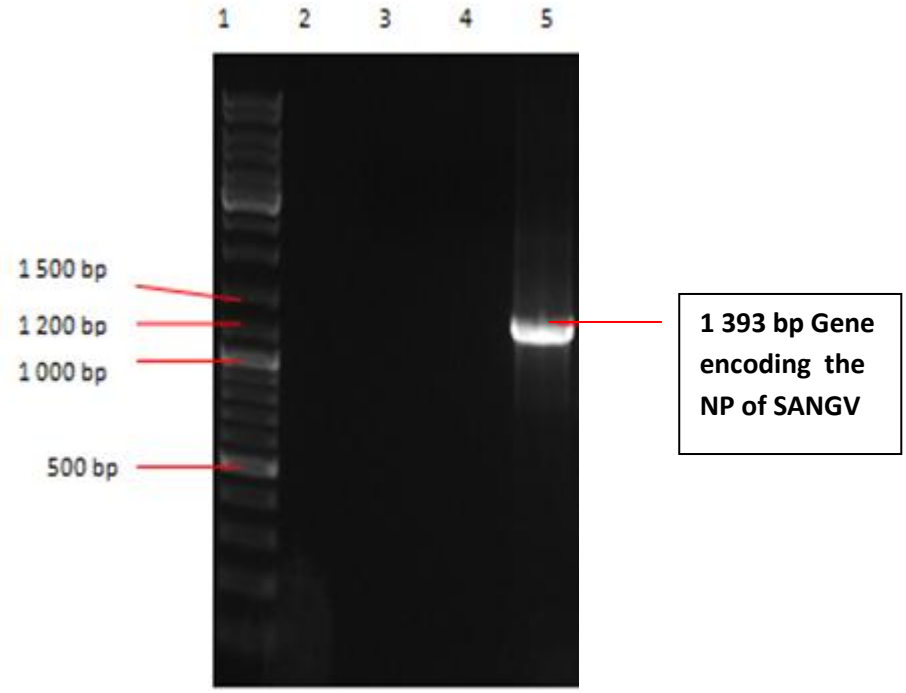

Figure 2.9: Confirmation of the S gene construct of SANGV after cells were transformed by using conventional PCR. Lane 1: O'Gene Ruler ™ molecular weight marker; Lane 2- negative control; Lanes 3 & 4- empty lanes; Lane 5- S gene of SANGV after amplification with T7 and BGH reverse primer

2.3.2 Nucleotide sequence analysis of the ORF of the S gene of SANGV in SANGV_pcDNA3.1 (+)

The sequence of the ORF of the S gene construct encoding the NP of SANGV was confirmed by Sanger sequencing. The sequencing data was edited using ChromasPro version 1.6 (Technelysium, South Brisbane, Australia) (Figure 2.10). The sequence was determined to confirm that the supplied SANGV_pcDNA 3.1(+) construct has the codon optimised S gene of SANGV that encodes the NP.

```
Sequencing      ---------ATGGCCACCCTGGAGGAGATCCAGAAGGAGATCAACATCCACGAGGGCCAG
Codon           GCCGCCACCATGGCCACCCTGGAGGAGATCCAGAAGGAGATCAACATCCACGAGGGCCAG
                          ************************************************

Sequencing      CTGATCATCGCCAAGCAGAAGGTGAAGGACGCCGAGAAGCAGTACGAGAAGGACCCCGAC
Codon           CTGATCATCGCCAAGCAGAAGGTGAAGGACGCCGAGAAGCAGTACGAGAAGGACCCCGAC
                ************************************************************

Sequencing      GACCTGAACAAGAGGGCCCTGAGCGACAGGGAGGGCATCGCCCACAGCATCCAGAGCAAG
Codon           GACCTGAACAAGAGGGCCCTGAGCGACAGGGAGGGCATCGCCCACAGCATCCAGAGCAAG
                ************************************************************

Sequencing      ATCGACGAGCTGAGGAGGCAGCTGGCCGACAGGGTGGCCGCCGGCAGGAACCTGGGCCAG
Codon           ATCGACGAGCTGAGGAGGCAGCTGGCCGACAGGGTGGCCGCCGGCAGGAACCTGGGCCAG
                ************************************************************

Sequencing      GAGAGGGACCCCACCGGCGTGGAGCCCGGCGACCACCTGAGGGAGAAGAGCATGCTGAGC
Codon           GAGAGGGACCCCACCGGCGTGGAGCCCGGCGACCACCTGAGGGAGAAGAGCATGCTGAGC
                ************************************************************

Sequencing      TACGGCAACGTGATCGACCTGAACCACCTGGACATCGACGAGCCCACCGGCCAGACCGCC
Codon           TACGGCAACGTGATCGACCTGAACCACCTGGACATCGACGAGCCCACCGGCCAGACCGCC
                ************************************************************

Sequencing      GACTGGCTGAGCATCGTGATCTACCTGACCAGCTTCGTGGTGCCCATCCTGCTGAAGGCC
Codon           GACTGGCTGAGCATCGTGATCTACCTGACCAGCTTCGTGGTGCCCATCCTGCTGAAGGCC
                ************************************************************

Sequencing      CTGTACATGCTGACCACCAGGGGCAGGCAGACCACCAAGGACAACAAGGGCATGAGGATC
Codon           CTGTACATGCTGACCACCAGGGGCAGGCAGACCACCAAGGACAACAAGGGCATGAGGATC
                ************************************************************

Sequencing      AGGTTCAAGGACGACAGCAGCTTCGAGGACGTGAACGGCATCAGGAAGCCCAAGCACCTG
Codon           AGGTTCAAGGACGACAGCAGCTTCGAGGACGTGAACGGCATCAGGAAGCCCAAGCACCTG
                ************************************************************

Sequencing      TTCCTGAGCATGCCCAACGCCCAGAGCAGCATGAAGGCCGAGGAGATCACCCCCGGCAGG
Codon           TTCCTGAGCATGCCCAACGCCCAGAGCAGCATGAAGGCCGAGGAGATCACCCCCGGCAGG
                ************************************************************

Sequencing      TTCAGGACCGCCGTGTGCGGCCTGTACCCCGCCCAGATCAAGGCCAGGAACATGGTGAGC
Codon           TTCAGGACCGCCGTGTGCGGCCTGTACCCCGCCCAGATCAAGGCCAGGAACATGGTGAGC
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Sequencing      CCCGTGATGAGCGTGATCGGCTTCATCACCCTGGCCAGGGACTGGACCGAGAGGATCGAG
Codon           CCCGTGATGAGCGTGATCGGCTTCATCACCCTGGCCAGGGACTGGACCGAGAGGATCGAG
                ************************************************************

Sequencing      AACTGGCTGGACCAGCCCTGCAAGTTCATGAGCGAGCCCAGCCAGACCAGCCTGCAGAAG
Codon           AACTGGCTGGACCAGCCCTGCAAGTTCATGAGCGAGCCCAGCCAGACCAGCCTGCAGAAG
                ************************************************************

Sequencing      GGCCCCGCCACCAACAGGGACTACCTGAACCAGAGGCAGGCCAGCCTGGCCCAGATGGAG
Codon           GGCCCCGCCACCAACAGGGACTACCTGAACCAGAGGCAGGCCAGCCTGGCCCAGATGGAG
                ************************************************************

Sequencing      ACCAAGGAGGCCCAGGCCGTGAGGCAGCAGGCCGTGGACGCCGGCTGCAACCTGGTGGAC
Codon           ACCAAGGAGGCCCAGGCCGTGAGGCAGCAGGCCGTGGACGCCGGCTGCAACCTGGTGGAC
                ************************************************************

Sequencing      CACATCGACAGCCCCAGCAGCATCTGGGTGTTCGCCGGCGCCCCCGACAGGTGCCCCCCC
Codon           CACATCGACAGCCCCAGCAGCATCTGGGTGTTCGCCGGCGCCCCCGACAGGTGCCCCCCC
                ************************************************************

Sequencing      ACCTGCCTGTTCATCAGCGGCATGGCCGAGCTGGGCGCCTTCTTCAGCATCCTGCAGGAC
Codon           ACCTGCCTGTTCATCAGCGGCATGGCCGAGCTGGGCGCCTTCTTCAGCATCCTGCAGGAC
                ************************************************************

Sequencing      ATGAGGAACACCATCATGGCCAGCAAGACCGTGGGCACCAGCGAGGAGAAGCTGAGGAAG
Codon           ATGAGGAACACCATCATGGCCAGCAAGACCGTGGGCACCAGCGAGGAGAAGCTGAGGAAG
                ************************************************************

Sequencing      AAGAGCAGCTTCTACCAGAGCTACCTGAGGAGGACCCAGAGCATGGGCATCCAGCTGGAC
Codon           AAGAGCAGCTTCTACCAGAGCTACCTGAGGAGGACCCAGAGCATGGGCATCCAGCTGGAC
                ************************************************************

Sequencing      CAGAGGATCATCATCATGTTCATGGTGGAGTGGGGCAAGGAGGCCGTGGACGGCTTCCAC
Codon           CAGAGGATCATCATCATGTTCATGGTGGAGTGGGGCAAGGAGGCCGTGGACGGCTTCCAC
                ************************************************************

Sequencing      CTGGGCGACGACATGGACCCCGAGCTGAGGAGCTTCGCCCAGGCCCTGATCGACCAGAAG
Codon           CTGGGCGACGACATGGACCCCGAGCTGAGGAGCTTCGCCCAGGCCCTGATCGACCAGAAG
                ************************************************************

Sequencing      GTGAAGGAGATCAGCAACCAGGAGCCCCTGAAGATCCACCACCACCACCACCACCACTAA
Codon           GTGAAGGAGATCAGCAACCAGGAGCCCCTGAAGATCCACCACCACCACCACCACCACTAA
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Figure 2.10: Nucleotideide sequence alignment. Sequence data of the modified NP gene aligned with codon optimized NP gene using Clustal W (www.genome.jp>tools>clustal w).

2.3.3 Transfection of BHK-21 cells using SANGV_pcDNA3.1(+) and positive control GFP

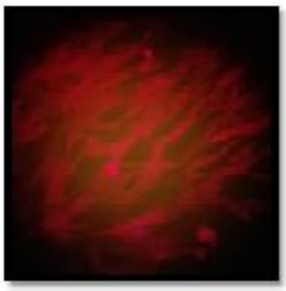
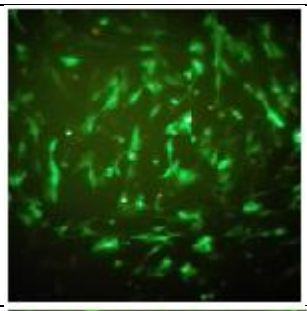
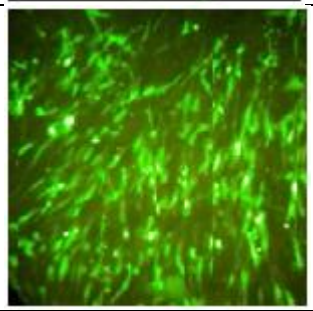
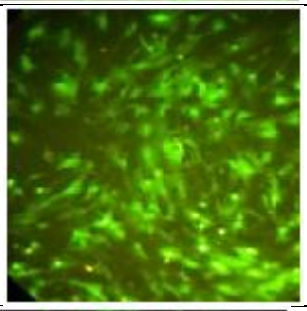
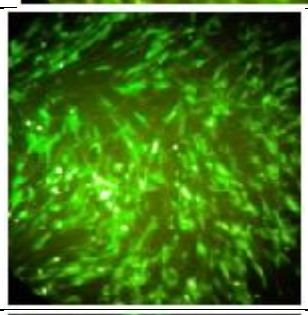
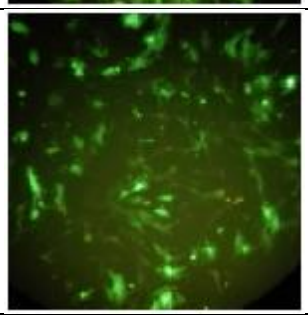
Transfected BHK-21 cells grown on coverslips were assayed 24 hours after transfection, from 24 well plates to allow sufficient expression of the protein. Subsequent to IFA, the cells were mounted on microscope slides and visualized under a fluorescent microscope using 100x magnification. BHK-21 cells transfected with the pcDNA3.1(+) encoding GFP and

SANGV_pcDNA3.1(+) construct were shown to express GFP and NP respectively at different DNA and lipofectamine concentrations (Figure 2.11). The results showed that transient expression of NP was optimal using a ratio of DNA(ug): Lipofectamine (µl) 2:0,75 and adding more DNA did not seem to increase the efficiency (Table 2.6). Hence 2:0,75 ratio was used for subsequent transfections of BHK-21 cells with SANGV_pcDNA3.1(+) construct. Expression of the NP of SANGV was confirmed by detection of the His6 tag using monoclonal anti-His antibodies and anti-mouse IgG conjugated to FITC. Evaluation of the transfection efficiency, which was based on fluorescing versus non-fluorescing cells by observation, was found to range from 50-60%.

The negative control consisted of non transfected cells where no DNA or transfection reagents were added. They were treated the same way as transfected cells in terms of changing of media after transfection and IFA conditions. For the positive control, cells were transfected with a plasmid expressing GFP. Transfection efficiencies as high as 90% were observed for GFP however, overall there was little difference observed when altering the ratio and hence it was decided to use a ratio of 2:0.75 for remaining experiments. This ratio reduced costs for lipofectamine reagent while maintain transfection efficiency.

Table 2.6: DNA:Lipofectamine ratios. Used to determine the optimal DNA concentration and lipofectamine ratio for transfection of BHK-21 using a pcDNA3.1(+) construct expressing GFP and SANG_pcDNA3.1(+).

DNA (µg):	0,5	0,5	2,0	2,0	3,5
Lipofectamine (µL):	0,75	1,5	0,75	1,5	0,75

DNA: concentration (µg)	Transfected BHK-21 cells:	Volume of reagents (µL):
1. Negative control (No DNA)		
2. Positive control: GFP (0,5µg)		P3000 (1,0µL) Lipofectamine 3000 (0,75µL)
3. Positive control: GFP (0,5µg)		P3000 (2,0µL) Lipofectamine (1,5µL)
4. Positive control: GFP (2,0µg)		P3000 (2,0µL) Lipofectamine 3000 (0,75µL)
5. Positive control: GFP (2,0µg)		P3000 (3,0µL) Lipofectamine 3000 (1,5µL)
6. Positive control: GFP (3,5µg)		P3000 (3,75µL) Lipofectamine 30000 (0,75µL)

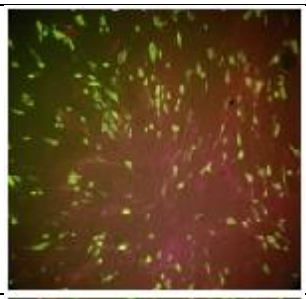
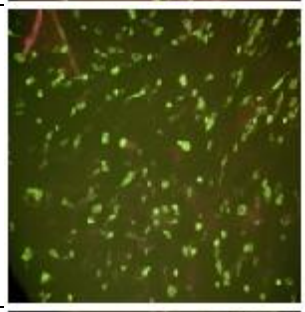
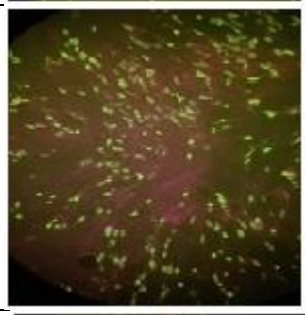
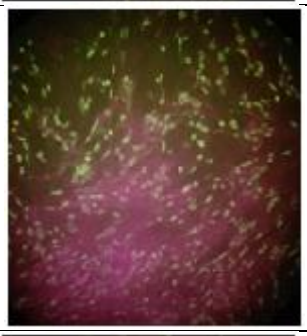
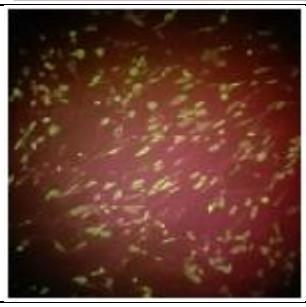
7. NP (0,5µg)		**P3000 (1,0µL)** **Lipofectamine 3000 (0,75µL)**
8. NP (0,5µg)		**P3000 (2,0µL)** **Lipofectamine 3000 (1,5µL)**
9. NP (2,0µg)		**P3000 (2,0µL)** **Lipofectamine (0,75µL)**
10. NP (2,0µg)		**P3000 (3,0µL)** **Lipofectamine 3000 (1,5µL)**
11. NP (3,5µg)		**P3000 (3,5µL)** **Lipofectamine 3000 (0,75µL)**

Figure 2.11: BHK-21 cells expressing the GFP and recombinant SANGV NP 18 hours post-transfection. Optimization reactions using different DNA concentration and varying volumes of the transfection reagents. 1) Negative control: non-transfected cells stained with Evans blue counter stain. 2-6) Positive control: cells transfected using 0,5, 2,0 and 3,0 µg of pcDNA3.1(+)_GFP expressing the recombinant GFP. 7-11) Recombinant antigen: BHK-21

cells transfected with SANGV_pcDNA3.1 (+) at varying DNA concentrations and reacted with monoclonal mouse anti-His antibodies targeting the His6 tag co-expressed with the recombinant NP of SANGV.

2.3.4 Analysis of the expressed recombinant protein in mammalian cells

An SDS-PAGE gel was prepared for analysis of the expressed recombinant protein after purification. A protein band of approximately 50 kDa was visible on the gel corresponding to the size of the native NP of SANGV. After purification, protein was collected in four 500µl eluates. Non-transfected cells, handled in the same manner as transfected cells, and purified using the Protino® Ni-TED 1000 columns (2.2.5) for purification of His6 tagged proteins were used to prepare mock antigen. No protein was detected in lane 2 from nontransfected cells. In lanes 3, 4 and 5 proteins of predicted size were detected. These proteins were expressed from the construct, SANGV_pcDNA3.1 (+) and detected using the His6 tagged recombinant NP of SANG as can be seen in lanes 3-5 in Figure 2.12.

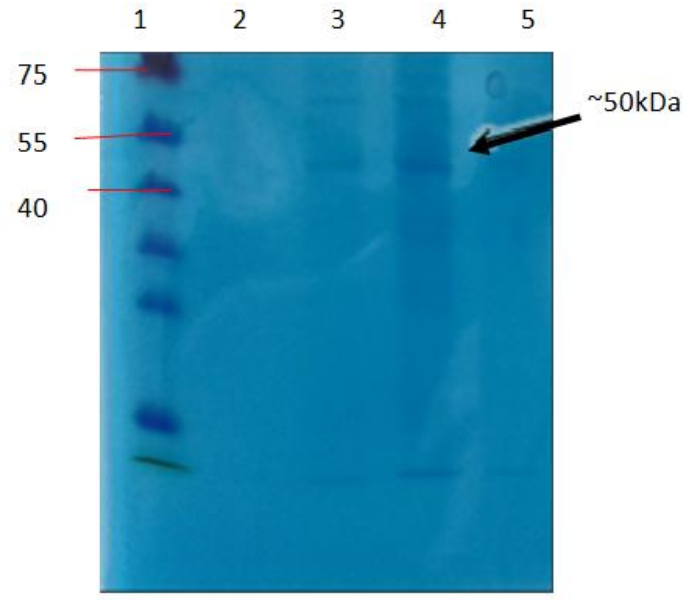


Figure 2.12: SDS-PAGE analysis of the expressed recombinant NP of SANGV in transiently transfected BHK-21 cells. Lane 1 shows the prestained protein ladder (10-180 kDa), Lane 2 is the negatve control where untransfected cells were lysed under the same condition as transfected cells, Lane 3-5 shows purified protein eluates from cells transfected with the SANGV NP construct where lane 4 shows the second eluate with a more distinct band.

2.3.5 Characterization of the recombinant SANGV NP using Western blot and anti-His monoclonal antibodies

The NP was collected in fractions after purification using Ni protino were separated on an SDS-PAGE gel and transferred to a PVDF membrane. The membrane was incubated with mouse monoclonal anti-His antibodies O/N to detect the His6 tag in the expressed recombinant NP of SANGV. A 50kDa band was visible on the film after developing and fixing. The band corresponds with the expected band of the native NP of SANGV. A prominent band from the second fraction in lane 3 was visible as can be seen in figure 2 .12 where lane 1 was loaded with prestained protein marker.

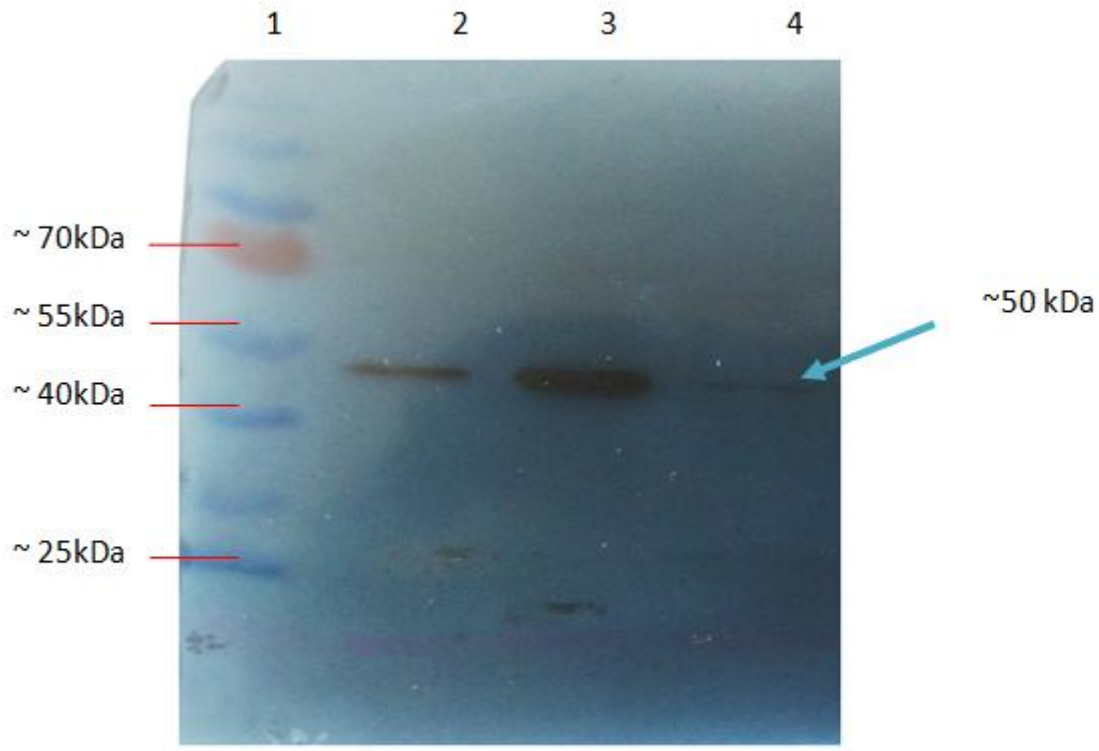


Figure 2.13: Western blot analysis of the recombinant NP of SANGV confirming the expression of an approximately 50 kDa protein. Lane 1: prestained protein ladder (10-180 kDa). Lane 2-4: Eluates of the purified recombinant protein.

2.4 Summary

Handling of hantaviruses requires high biocontainment facilities such as biosafety level BSL 3 and 4 laboratories. For diagnosis and surveillance of hantaviruses, serological assays are frequently employed. Commonly used assays include IgM and IgG ELISA as well as immunoblots and neutralization tests which are often laborious and take a long time to

perform in the event where rapid diagnosis of hantaviruses is needed (Machado et al., 2009). To overcome the requirement for a high biosafety facility for preparing viral antigens and in order to perform hantavirus surveillance, a recombinant NP of SANGV was synthesized that could be used as a safe tool in serological assays.

The NP of hantaviruses is comprised of 428-433 aa residues and the difference is a result of the varying length of the 3' NCR (Schlegel et al., 2014). The nucleotide and aa identity of the ORF of the NP of hantaviruses ranges between 54-74% and 46-83% respectively (Schlegel et al., 2014). Phylogenetic analysis of all three segments of SANGV revealed close relation to the European DOBV (Klempa et al., 2012). The NP has been found to be antigen of choice for diagnosis of hantaviruses because it induces an early and lasting immune response (Hedman et al., 1991; Sjolander et al., 1997). A level of cross-reactivity of the NP among closely related hantaviruses has been shown to exist. The first 100 aa on the N termini have been found to be cross reactive whereas for a more specific diagnosis work using truncated NPs have shown serotype specific epitopes (Meisel et al., 2006; Yoshimatsu et al., 2002; Yoshimatsu et al., 2014).

The use of a mammalian system for expression of protein as opposed to the frequently used bacterial expression allows proper folding and post translation modifications of recombinant proteins. The limitation, however, includes low protein yields and is quite costly compared to bacterial expression. Highly pure, phenol, salt and endotoxin free plasmid DNA is a requirement for transfection of mammalian cells as these could damage cells and interfere with the lipids thereby decreasing transfection efficiency. An endotoxin removal plasmid purification kit was therefore used for this purpose.

The gene encoding the NP of SANGV was synthesised and supplied in the mammalian expression vector pcDNA3.1 (+). The gene was designed with a few modifications and among these was the EcoRI and XbaI sites that are present on the expression vector to facilitate cloning. A Kozak sequence was added at the 5' end of the gene and this was done to facilitate initiation of translation of the recombinant protein in a mammalian cell. The sequence was codon optimized for expression in mammalian cells.

To reduce the use of tandem rare codons, prolong the half-life of mRNA, improve ribosomal binding, increase stability of mRNA and modify negative cis-acting sites
the codon adaptation index, frequency of optimal codons and GC content was improved.

Chemically competent *E.coli* cells were transformed using the construct that was designated SANGV_pcDNA3.1 (+). A PCR and sequencing reaction was performed to confirm the presence of the S gene of SANGV in pcDNA3.1 (+). Under the control of the CMV promoter, the NP of SANGV was expressed in transfected cells. Initial confirmation of the expressed recombinant protein was done by immunofluorescence assay using monoclonal anti-His antibodies that targeted the C-terminus His6 tag on the protein. The protein was characterised by separation on an SDS-PAGE gel and Western blot and a protein of approximately 50 kDa was observed corresponding to the size of the native NP of SANGV.

At 2:0.75 ratio of DNA: Lipofectamine, optimal expression of the recombinant SANGV NP was obtained using the 24 well plate format. This ratio was selected and used when scaling up the transfection experiments to prepare antigen slides for immunofluorescent antibody assays and recombinant antigen for in house ELISA in Chapter 3.

Chapter 3: Application of SANGV recombinant NP as a tool for serological detection of IgG antibody against hantaviruses in human sera

3.1 Introduction

Diagnosis of HFRS and HCPS in humans and surveillance of at risk populations relies predominantly on serological testing. Due to the short-lived viremia during acute phase of infection, RT-PCR is not routinely used (Sjölander & Lundkvist, 1999). Traditional methods such as IFAs have been utilized for hantavirus serological testing but are less sensitive when compared to ELISA based on native NP antigens (Sjölander & Lundkvist, 1999; Meisel et al, 2006). Due to an early and long lasting immune response, the NP of hantaviruses has been the recombinant antigen of choice for serological diagnosis of hantaviruses (Sjolander et al., 1997; Hedman et al., 1991). The NP of hantaviruses is highly immunogenic in reservoir hosts, immunised laboratory animals as well as infected humans (Meisel et al, 2006).

Detection of IgM and IgG against hantaviruses form the basis of diagnosis and surveillance using different formats of ELISA and Western blot (Hujakka et al., 2003; Sirola et al., 2004). Viral antigens from hantavirus infected cells or recombinant N proteins are used in serological assays; recombinant NPs are used because as it has been shown that neutralizing NP specific antibodies occur early after infection and probably persist for life whereas antibodies against surface protein GP can be found in the late convalescent sera. The NP of hantaviruses can be expressed in bacteria, yeast, insect and mammalian cells. The high level of amino-terminal conserved regions on the NP among members of the hantavirus genus means that cross-reactivity between distantly related hantaviruses occurs; thus allowing for the detection of a majority of hantavirus infections. Heterologous antigens can be found with most commercially available hantavirus ELISA kits. A species-specific carboxyl terminal region for diagnosis exists should it be required for example in areas where more than one hantavirus occurs and may cause diseases with different severity (Elgh et al, 1998; Sjölander & Lundkvist, 1999; Meisel et al, 2006).

The NP is highly conserved and cross-reactive epitopes exists on the N-terminal located within the first 100 aa (Elgh et al., 1998; Gott et al., 1997; Yamada et al., 1995). Sera collected from patients presenting with HFRS recognised a 1-119 aa residue fragment of the NP protein from a PUUV serotype and HNTV. There appears to be less conserved regions at the C terminal region of the NP as shown using serotype specific monoclonal antibodies

which identified epitopes that have been located in the C-termini half of the NP (Yoshimatsu et al., 1996).

The initial assumption was that one NP antigen from the hantavirus groups such as HNTV/ Seoul/ DOBV and PUUV/Tula as well as SNV/ ANDV could be sufficient for detection of either one hantavirus species as a result of the NP cross reactivity. Other studies emphasize the use of a homologous NP antigen for a highly sensitive detection method especially in areas where hantavirus infections are endemic and IgG prevalence is high in the population (Bi et al., 2008; Zoller et al., 1995; Vapalati et al., 1996; Meisel et al., 2006).

The cross-reactivity, or lack thereof, of immune sera of mice vaccinated with full-length NP cDNA of homologous viruses (PUUV, SEOV, SNV) was shown to be highly sensitive against full and truncated NPs and a similar observation with human serum samples was made. Major immune responses are directed towards the amino terminal regions of the NP and less towards the carboxyl end. It has been hypothesized that the region recognised at first exposure to hantaviruses will determine the profile of immune sera in B-cell epitope differentiation. Observations of greater cross-reactive characteristics of neutralizing antibodies are seen during the acute or early convalescent stages in infection rather than in sera collected during late convalescent stages when there was less cross reactivity of neutralising antibody (Lundkvist et al., 2007).

In this chapter, the application of a recombinant NP for serology was explored by making use of the recombinant SANGV NP in serological tests including in-house prepared antigen slides with cells expressing the protein. An attempt was made to prepare an in house ELISA by coating plates with purified recombinant NP. Finally, sera from patients with fever of unknown origin from the Free State region were tested for antibody against African, Asian and European hantaviruses.

3.2 Methods and Materials

3.2.1. Serum samples

A total of 176 serum samples from patients with fever and no diagnosis were tested retrospectively for antibody against hantaviruses. The sera were submitted to theDepartment of Medical Microbiology and Virology, NHLS Universitas, between 2008 and 2011. The samples were stored at -20^{o}C.

The samples were tested for IgG antibody against hantaviruses by IFA using antigen slides prepared from transiently transfected cells and commercially available ELISA kits. In total there were 176 samples and each was assigned a laboratory number (Table 3.5). For some samples insufficient sample was available for testing using commercial ELISA.

3.2.2 Preparation of transfection grade SANGV_pcDNA3.1 (+) plasmid DNA

Purified SANGV_pcDNA3.1(+) plasmid DNA was prepared from O/N culture inoculated with glycerol stock and purified using the Qiagen ® Plasmid mini kit (Qiagen, Hilden, USA) according to manufacturer's instructions. Briefly, a 5 ml aliquot of media containing amp was inoculated with glycerol stock from bacterial culture prepared from positively transformed cells. The cells were allowed to grow O/N and thereafter harvested by centrifugation at 16,000 xg for 15 minutes. The cell pellet was resuspended in 0,3ml of buffer 1 (supplied in the kit and prepared by adding RNase solution and stored at 2-8°C). A 300 µl aliquot of buffer 2 was added and mixed by vigorously inverting the tube 4-6 times and incubated for 5 minutes at RT. A 300 µl aliquot of pre-chilled buffer P3 was added and mixed by vigorously inverting the tube 4-6 times and the tube was then incubated on ice for 5 minutes. The tube was centrifuged at 14,000 xg for 10 minutes at 4°C. A QIAGEN-tip 20 was equilibrated by applying 1 ml of buffer QBT and allowed to empty by gravity flow. The cleared cell supernatant was then applied to the tip and washed 2×2 ml with buffer QC. Plasmid DNA was eluted by adding 800 µl of buffer QF and precipitated by adding 560 µl of RT isopropanol and centrifuging at 15,000 xg for 30 minutes at 4°C. The pellet was washed with 1 ml RT 70% ethanol and centrifuged at 15,000 xg for 10 minutes. The pellet was air dried and re-dissolved in 30 µl of NFW and thereafter the concentration and purity of the DNA was determined using the NanoDrop 2000 spectrophotometer

Larger preparations of the purified plasmid DNA were achieved by using the Qiagen® Plasmid *Plus* Midi kit (Qiagen, Hilden, USA). The high yield protocol was used. Briefly, from a starter culture of 5 ml, a 25 ml culture was prepared by inoculating 50µl of the starter media and shaking (300rpm) at 37^{o}C for 12-16 hrs. The cells were harvested by centrifuging at 6000xg for 15 minutes at 4^{o}C. The cell pellet was completely resuspended in 4 ml buffer P1 the tube. A 4ml aliquot of buffer P2 containing the LyseBlue reagent was added and mixed by inverting so that the solution turned a homogeneous blue colour. Pre-chilled buffer S3 was added at 4ml aliquot to the tubes and mixed by inverting until the solution is colourless.

The lysate was applied to the QIAfilter Cartridge and incubated at RT for 10 minutes. By inserting the plunger into the cartridge, the cell lysate was filtered into the tube. An aliquot of 2 ml buffer BB was added to the cleared lysate and mixed by inverting 6 times. The lysate was transferred to a QIAGEN Plasmid Plus spin column through tube extenders. Vacuum (≈300 mbar) was applied until the liquid has been drawn through the column. Buffer ETR, at an aliquot of 0.7 ml, was added to wash the DNA and the liquid was drawn by vacuum. Further washing was performed. Residual wash buffer was removed by centrifuging the column briefly at 10 000 xg. The spin column was placed into a clean microcentrifuge tube and NFW was added to elute the DNA.

3.2.3 Preparation of antigen slides using transfected cells and screening of human serum samples

BHK-21 cells were transfected using the Lipofectamine® 3000 reagent. Screening for the expression of the recombinant NP of SANGV was performed by seeding and transfection of cells in 24-well plates as previously described in 2.2.4. In addition, cells were seeded in 6-well plates at a seeding rate of 5×10^{5} cells/$cm^{2.}$ The cells were allowed to reach 70-90 % confluency before transfection. BHK-21 cells were transfected in 6 well plates using the optimal ratio of 2:0,75 DNA : Lipofectamine as determined in 2.3.3 by diluting 9µg of the SANGV pcDNA3.1(+) construct and 13,5µl of P3000 in 250µl of Optimem™ and incubating with diluted 6,75µl of Lipofectamine™ 3000 reagent in 250µl Optimem™ to allow for complexes to form. The Lipofectamine/DNA mix was incubated for 15 minutes and inoculated into plates where cells were growing.

The cells were harvested from 6 well plates 24 hours post-transfection. BHK-21 cells were harvested by trypsinization of the cells. Briefly, transfection medium was discarded and cells were washed with PBS. An aliquot of 100µl of 10x typsin (Lonza, Verviers, Belgium) was added to each well and incubated at 37°C for 5 minutes. Trypsin was neutralized by addition of fresh growth media, which was used for cell resuspension. The resuspended cells were centrifuged at 2000 xg. The pellet was resuspended in 500µl of 1XPBS supplemented with 10% FBS to facilitate attachment of cells to the surface of the 8 well glass slides. A 10µl aliquot of cell suspension was applied to each well of an 8 well slide and cells were dried at 37°C. The cells were fixed and permeabilized by immersing in a cold solution of acetone at -20°C for 20 minutes. The cells were washed twice with 1xPBS, air dried and slides were stored at -20°C until use.

Human serum samples from patients in the Free State with febrile illness and no diagnosis submitted to the laboratory for routine diagnostic tests and stored at -80°C, were screened for IgG antibody against hantaviruses. The slides were allowed to reach RT before applying 10µl of patient serum diluted at 1:10 in blocking buffer and incubating the slides at 37°C for 20 minutes in a humid environment. The slides were washed 3 times using 1xPBST and 10µl of goat anti-human IgG conjugated to FITC (KPL, Maryland, USA) diluted at 1:100 in 0.1% Evans blue, was applied. The slides were incubated for 20 minutes and washed with 1xPBST. The cells were air-dried and mounted with a cover slip using glycerol mounting media.

3.2.4 Preparation of an in-house ELISA using the recombinant NP of SANGV

An in-house ELISA was attempted using the recombinant NP of SANGV produced from transfected BHK-21 cells. Briefly, 5xT75 flasks were seeded with BHK-21 cell at $3{,}75\times10^6$ cells. For a single T75 flask, 56,25µl of lipofectamine 3000 reagent was diluted in 937,5µl of OptiMEM. While incubating at RT 37,5µg of SANGV_pcDNA3.1(+) was diluted in 937,5µl and 112,5µl of P3000 reagent was added to the same well. The diluted DNA/P3000 was added to the dilute Lipofectamine 3000 and incubated at RT for 15 minutes to allow complexes to form. The growth media was replaced with antibiotic free media and the complexes were added. The cells were kept at 37°C in a CO_2 incubator for 48 hours to allow for sufficient protein expression. The flasks were removed from the incubator and the cells were lysed using BSA lysis buffer (Appendix B) while shaking on ice for 30 minutes. The lysate was kept at -20°C until further use.

The protein was purified as in 2.2.5 and fractions of 500µl volume were collected and designated eluates 1 to 4. The protein concentration for each fraction was determined using the Qubit® protein assay kit (Invitrogen,USA) according to manufacturers' instructions. The Qubit® Protein Assay kit is supplied with three standards. The Qubit fluorometer was calibrated for each measurement using the three standard solutions supplied. The protein concentration was recorded in µg/ml. The ELISA was performed in a 96 well immunoassay plate, and optimal working dilutions of the reagents were determined by checkerboard titrations. Throughout the assay, reagent volumes of 100µl were used, the diluent for reagents was PBS containing 2% skimmed milk powder, incubations were performed for 1 hour at 37°C, wells were blocked after coating with 200µl PBS containing 10% skimmed milk

powder and plates were washed thrice with PBS containing 0.1% Tween 20 (PBST) unless specified.

Briefly, a 96 well microtiter Polysorb plate (Nunc Immunoplate, Denmark) was coated O/N at 4°C with recombinant SANGV NP and mock antigen. Antigen was applied at dilutions from 1:10 to 1:320. Mock antigen from untransfected BHK-21 cells treated the same way as transfected cells was used to coat microtiter plates. The plates were washed 3 x 15 seconds using 0,1% PBST and blocked with 200µl of 10% skimmed milk diluted in PBS and incubated for 1 hour at 37°C. The plate was washed and human positive and negative controls from the commercial assay kits were reacted for 1 hour at 37°C. Human positive serum samples (G5 and G4) were used at a 1:100 dilution in 2% skimmed milk diluted in PBS. Plates were washed as previously described and goat antihuman IgG conjugated to HRPO (Zymed Laboratories, Cardiff, United Kingdom) diluted 1:2000 was applied to each well. After incubation at 37°C for 1 hour and washing, reactors were visualized using the 2,2'-azino diethyl-benzothiazoline-sulfonic acid peroxidase substrate (ABTS) (Kirkegaard and Perry Laboratories, Merryland, United States of America). The plates were incubated at room temperature (22-25°C) in the dark and the OD values were read at 405 nm with reference wavelength at 650nm. The net OD value of each test and control serum sample was determined. Attempts to optimise the ELISA included concentrating the antigen by reducing the volume on the speedivac and then coating plates with higher concentrations of the antigen and using lower dilutions of detection antibody.

3.2.5Screening of serum samples using commercially available anti-hanta ELISA kits

Two commercial ELISA kits (EUROIMMUN, Luberck, Germany) for the detection of antihantavirus IgG antibodies were used in the study to test human serum samples from patients with unknown causes of fever. The commercial ELISA can provide a semi quantitative or quantitative *in vitro* assay for human antibodies (IgG) against hantavirus strains of the "Eurasian" pool that includes HNTV, DOBV and PUUV strains. The "American" kit was comprised of a mixture of ANDV and SNV NP antigens. The plates are precoated with a mixture of the recombinant NP antigens. A specific IgG antibody binds to the antigen mixture for a positive sample and upon addition of the conjugated secondary antibody (antihuman IgG) and substrate, a colour change can be observed and the reaction can be stopped.

The kit was allowed to reach RT before the assay was performed. Serum samples were diluted 1:100 in sample buffer supplied with the kit and each sample added to a well in the microplate. The kit was supplied with a positive control, negative control and three calibrators (Calibrator 1, 2 and 3). For a semi-quantitative assay, only calibrator 2 was used as per the package insert along with the positive and negative controls which are supplied ready for use. Calibrator 2 contains 20 relative units (RU) per ml and this range is the recommended cut-off value for the upper limit of negative samples. Unless otherwise stated, a 100µl volume of each reagent was used. The plates were covered with protective foil and samples were incubated at 37^{o}C for 60 minutes. The plates were washed using a Tecan 96 well plate washer. Plates were washed three times with 450µl of the working solution of wash buffer supplied with the kit. Peroxidase labelled antihuman IgG supplied in the kit was added and the plate was incubated at RT for 30 minutes. Plates were washed and TMB substrate solution was added into each microplate well and incubated at RT for 15 minutes and the stop solution was then added. The results were read within 30 minutes of adding the stopping solution and the OD readings were taken at 450nm wavelength with 620nm as reference. Results were calculated according to the given formula in the kit for a semi quantitative assay.

3.2.6. Controls for in-house assays

In the absence of confirmed SANGV IgG positive samples for the development of the in-house assays, and assuming that there is some degree of serological cross reactivity against heterologous antigens as shown by previous reports, positive controls from the commercial ELISA were used as controls for the in-house IFA and ELISA. In total there were six commercially available ELISA kits each containing positive controls from immune human sera against antigens from Europe and Asia, and America. Undiluted controls from the commercial kits were tested for reactivity against the recombinant SANGV using transiently transfected cells fixed on slides. Positive reactors were identified and subsequently used as positive controls each time an IFA was run and were used to attempt to optimise the ELISA.

3.3 Results

3.3.1 Immunofluorescence assay using recombinant SANGV_NP antigen slides

Antigen slides prepared from transiently transfected cells were used for detection of antibodies against hantavirus using recombinant SANGV NP antigen. Expression of

recombinant SANGV was confirmed using antihistidine IFA assays and then using controls obtained in commercial ELISA kits. Human sera were subsequently screened as described in the methods. Controls from six commercial ELISA kits for both Eurasian (Europe and Asia) and American used in 3.2.4 were tested for reactors against the recombinant NP of SANGV using BHK 21 cells transiently transfected with SANGV_pcDNA3.1(+) DNA construct. Positive controls designated one, four and six were taken from the Euroimmun anti-hanta virus pool 2 "American " ELISA IgG kit and positive controls designated two, three and five were taken from the anti-hanta virus pool 1 "Eurasia" ELISA IgG kits. The controls were applied undiluted on the IFA slide. Controls one, two and four reacted with the transiently transfected cells showing positive fluorecesence. Control serum three was inconclusive and controls five and six were negative. A positive reactor, control two, from the anti-hanta virus pool 1 kit was selected and used as a positive control for screening human sera for antibody against SANGV by IFA and optimising the ELISA.

A total of 176 human sera were screened using transiently transfected cells fixed on glass slides, 4/176 were positive by IFA using recombinant NP of SANGV as shown in Table 3.5.

3.3.2 In-house ELISA assay

Recombinant NP was purified from the supernatant of transfected and untransfected cells after lysis using Net/BSA buffer. The protein was collected in four eluates (designated eluate 1 to 4) of 500µl each and the protein concentrations quantified using Qubit as shown in Table 3.1.

Table 3.1: Protein concentrations of recombinant NP fractions after purification

Eluate	Concentration (µg/ml)
1	10,2
2	15,2
3	14,0
4	13,1
Mock	5,64

An in-house ELISA using recombinant NP of SANGV was performed using controls and calibrators from the commercial kits and two serum samples that were positive in IFA. A serial dilution of the NP was prepared from 1:10, 1:40, 1:160 and 1:320 in PBS and each dilution used to coat wells overnight. Wells coated with PBS only were used as negative

controls (Table 3.2). Mock antigen was prepared from untransfected cells that were treated in the same manner as transfected cells. The mock antigen was diluted 1:10 and used to coat wells overnight. Example of plate layout is shown in Figure 3.1. In some instances, onlyPBS or only mock antigen coated wells were included and not both (Table 3.3).

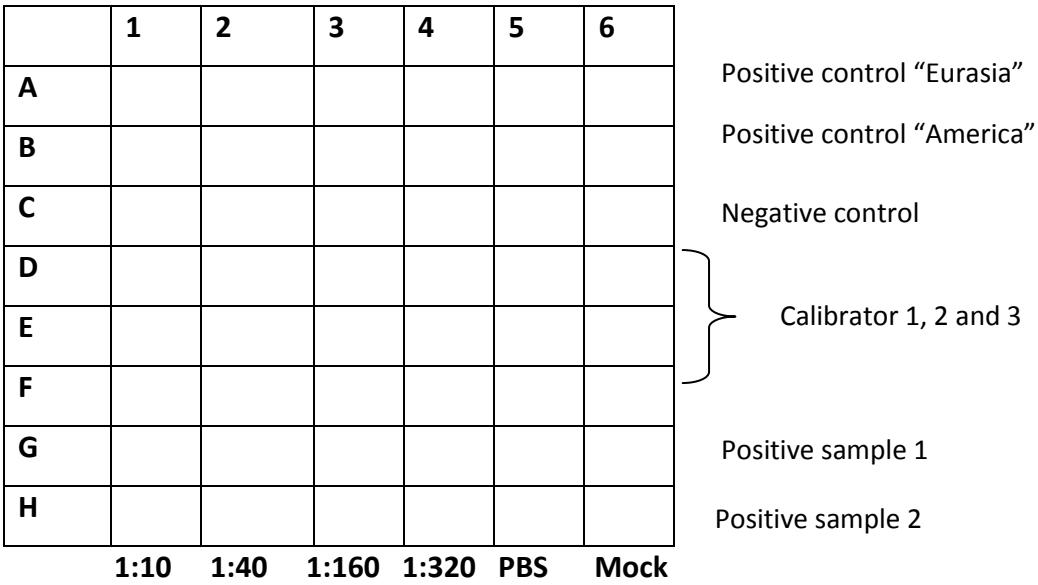


Figure 3.1: In-house ELISA plate layout

Table 3.2: **ELISA results from plate coated with recombinant NP of SANGV**. Columns 1 to 4: OD results from wells coated with recombinant NP of SANGV at 1:10; 1:40; 1:160; 1:320 respectively. Column 5: OD results from wells coated with PBS. Rows A and B are results using positive controls from the Eurasian hantavirus commercial kit and American hantavirus kit respectively. Row C is the results from a negative control from the commercial kit. Rows D to F are results from three calibrators from the Eurasian kit. Rows G and H are results from patient sera that reacted positively using the IFA.

	1	2	3	4	5
A	2.0350	2.3250	2.4260	2.4090	2.1570
B	1.7110	1.9900	2.0560	1.8240	1.6240
C	3.0790	3.1840	2.9520	2.9520	2.4780
D	0.7490	0.8530	0.6850	0.5780	0.4400
E	0.1860	0.2080	0.1180	0.0980	0.0790
F	1.7030	1.7990	1.8640	1.5770	1.3710
G	1.3460	1.5440	1.6200	1.4480	1.7350
H	0.9950	0.9310	1.1160	0.8610	1.3030

Table 3.3: ELISA results obtained using recombinant NP of SANGV. Columns 1 to 3: NP diluted 1:10, 1:160 and 1:320. Column 4: undiluted mock antigen Rows A and B: positive controls from the Eurasian and American hantavirus kits. Row C: negative control sera from the hantavirus commercial kit. Rows D,E F: calibrators from the Eurasian kits

	1	2	3	4
A	1.4090	0.1140	1.8960	2.7240
B	1.5070	0.0800	2.0870	2.9350
C	1.7050	0.0990	1.9460	2.9770
D	0.0470	0.0160	0.0590	0.0930
E	0.0470	0.0120	0.0540	0.1010
F	0.1670	0.0140	0.1610	0.1530

In attempts to optimise the ELISA the plates were coated with recombinant antigen at lower dilutions. The results are shown in table 3.4 where undiluted antigen from purification step was coated to the plate and control sera was used. Eluate one in column 1 contains 10µg/ml of protein and eluate 2-4 contain 15,4; 14,0 and 13,1µg/ml of protein respectively.

Table 3.4: ELISA results obtained from plate coated undiluted recombinant NP of SANGV. Columns 1 to 4: antigen from purified eluates 1-4. Column 5: PBS. Rows A and B: positive control from Eurasian hantavirus kit and American kit respectively. Row C: negative control from hantavirus kit.

	1	2	3	4	5
A	0.4290	0.4200	0.3530	0.3080	0.0850
B	0.4260	0.3990	0.3510	0.2990	0.0820
C	0.6300	0.5620	0.4690	0.4760	0.1410

To differentiate between negative and positive samples net OD values are calculated and a cut off value determined based on negative controls.

Net optical density (OD):

Net OD = OD in wells with recombinant antigen minus OD in wells with mock antigen or PBS

Cut off value is usually mean net OD negative controls + 2 standard deviations

However, there was no differentiation between positive and negative controls. In some instances, the net OD values of negative samples were greater than positive controls. In general, at high dilutions of recombinant antigen, the OD values of samples reacted with PBS were lower than compared with the OD values of the same samples reacted with recombinant or mock antigen. This suggests a non-specific reaction of the serum (controls) with the cell preparation (recombinant NP or mock) resulting in the high OD values. However when the recombinant NP was diluted to reduce the non specific reaction although the OD values were lower (less non specific reactivity) there was still no differentiation between the net OD values for positive and negative samples. These results strongly indicate that the amount of recombinant antigen was too low for reactivity. Yield of protein from mammalian expression can be low and is a problem when preparing sufficient antigen for ELISA.

3.3.3 Commercial ELISA for detection of anti-hantavirus IgG in human sera

To screen the cohort of human sera using commercial ELISA a semiquantitative ELISA was performed. The results were calculated based on the extinction value of calibrator 2 (Appendix C). After the OD value of each sample was measured, it was divided with the value of calibrator 2. A ratio of <0,8 was regarded as negative, ≥ 0,8 < 1,1 was regarded as borderline and values ≥ 1,1 were regarded as positive. Although 176 sera were tested by IFA there was an insufficient amount of sample to test using the ELISA. The number tested is indicated in the results. A total of 6/165 were positive by hantavirus pool 1 commercial ELISA kit with antigens from Europe and Asian hantaviruses and 5/165 were borderline. A total of 3/157 samples were positive using the hantavirus pool 2 commercial ELISA kit with antigen from American hantaviruses, and 2/157 were borderline (Table 3.5).

Table 3.5: Commercial ELISA and IFA results. Results obtained from screening human serum samples using IFA and recombinant SANGV NP and ELISA using two commercial kits with a mixture of recombinant NP of hantaviruses from Europe, Asia (Eurasia) and America (America). Inconclusive IFA results are indicated with an X. N/T are samples not tested.

Sample lab no:	IFA results:	ELISA results: "Eurasia"	ELISA results: "America"
C1	-	-	-
D2	-	-	-
E2	-	-	-
D3	-	-	-
E3	-	-	-
G4	-	-	-
A5	-	-	-
F5	X	-	-
G5	+	-	-
A6	-	-	N/T
C6	-	N/T	N/T
D6	-	N/T	N/T
E6	-	-	-
A1	-	-	-
B1	-	-	-
C1	-	-	-
E1	-	-	-
F1	-	+	-
G1	-	-	-
A2	X	+	-
C2	-	-	N/T
E2	-	N/T	N/T
G2	-	-	-
C3	-	Borderline	Borderline
A4	-	-	-
C4	-	-	-
D4	-	-	-
F4	-	-	-
B1	+	-	-
G2	-	-	-

B3	-	-	-
E3	-	-	N/T
F3	-	N/T	N/T
G3	-	+	-
A4	-	-	-
C4	-	-	-
D4	-	-	-
E4	-	-	-
B8	-	-	-
I8	-	-	-
J8	-	-	-
A1	+	-	-
D1	-	-	N/T
F1	-	N/T	N/T
G1	-	-	-
H1	-	-	-
E2	-	-	-
B3	-	+	Borderline
C3	-	-	-
D3	-	-	-
E3	-	-	-
G4	+	-	-
H4	X	-	-
A5	-	-	N/T
F5	-	N/T	N/T
A7	-	N/T	-
H7	-	-	-
A6	-	-	-
27/9/22	-	-	-
27/9/11	-	-	-
29/7/4	-	-	-
29/7/6	-	-	-

27/9/9	X	-	-
27/9/23	-	-	-
27/9/30	-	-	N/T
27/9/26	-	N/T	N/T
27/9/29	-	-	N/T
2/8/9	-	Borderline	-
2/8/15	-	-	-
2/8/13	-	-	-
2/8/3	-	-	-
2/8/10	-	-	-
27/8/8	-	-	-
27/8/11	-	-	-
27/8/9	-	-	-
2/8/24	-	-	N/T
27/8/6	-	N/T	N/T
58/8/3	-	-	N/T
58/8/2	-	N/T	-
58/8/1	-	-	-
58/8/5	-	-	-
58/8/6	-	-	-
58/8/8	-	-	-
10/10/21	-	-	-
24/11/1	X	-	-
10/10/29	-	-	-
10/10/27	-	-	N/T
10/10/26	**-**	**N/T**	**N/T**
10/10/25	-	-	-
24/11/07	-	-	-
24/11/16	-	-	-
24/11/5	-	-	-
24/11/3	-	-	-
70/9/13	-	-	-

70/9/6	-	-	-
70/9/5	-	-	-
70/9/4	-	-	-
70/9/1	-	-	-
70/9/3	-	-	-
70/9/7	-	-	-
70/9/12	-	-	-
70/9/11	-	-	-
70/9/18	-	-	-
70/9/17	X	-	-
72/09/5	-	-	-
70/9/15	-	-	-
39/9/7	-	-	-
39/9/6	-	-	-
39/9/5	-	-	-
39/9/3	X	-	-
39/9/2	-	-	-
39/9/1	-	-	-
39/9/14	-	-	-
39/9/13	-	-	-
39/9/12	-	-	-
39/9/11	-	-	-
39/9/10	-	-	-
39/9/9	-	-	-
58/8/13	-	-	-
58/8/19	X	-	-
58/8/11	-	-	-
58/8/20	-	-	-
58/8/34	-	-	-
58/8/33	-	-	-
58/8/31	-	-	-
10/10/3	-	-	-

58/8/39	-	-	-
58/8/38	-	-	-
24/10/4	-	Borderline	-
10/10/7	-	Borderline	-
10/10/9	-	-	-
10/10/10	-	Borderline	-
4/10/4	-	-	+
4/10/5	-	-	-
4/10/6	-	-	-
4/10/7	-	-	-
58/8/37	-	-	-
10/10/19	-	-	-
10/10/18	-	-	-
10/10/17	-	-	
10/10/16	-	-	-
10/10/15	-	-	-
10/10/13	-	+	-
10/10/5	-	-	+
10/10/6	-	-	-
44/10/3	-	+	-
44/10/4	-	-	-
44/10/5	-	-	-
24/10/7	-	-	-
10/10/23	X	-	-
10/10/24	-	-	-
10/10/30	-	-	-
10/10/32	-	-	-
45/10/7	-	-	-
45/10/3	-	-	-
45/10/4	-	-	-
45/10/5	-	-	-
45/10/1	-	-	-

44/10/2	X	-	-
45/10/2	-	-	-
10/10/31	-	-	-
60/9/1	-	-	-
45/10/16	-	-	-
45/10/8	-	-	-
45/10/9	-	-	-
45/10/10	-	-	-
45/10/11	-	-	-
45/10/12	-	-	-
45/10/13	-	-	-
60/9/6	-	-	-
24/10/01	-	-	-
24/10/2	-	-	-
24/10/3	-	-	-
45/10/14	-	-	+
45/10/15	-	-	-
60/9/3	-	-	-

3.4 Summary

ELISA is a plate based technique used for detecting and quantifying proteins and antibodies. An antigen such as the recombinant NP of SANGV is immobilised on the surface of the plate and antibodies are added. Indirect IgG and M-antibody capture ELISA are the most commonly used tests in serology for hantaviruses. An ELISA assay has the advantage of being highly specific. Serological cross-reactivity among hantaviruses in ELISAs is not uncommon due to the amino terminal of the NP which have shown to increase with time after onset of illness (Elgh et al., 1998; Lundkvist et al., 1999). Unlike IFA which are hard to standardize and are dependent on the observer, ELISA can be automated and compared and are useful for large scale surveillance (Bayer et al., 2001). For the development of in-house assays, positive controls from commercial ELISA were used as controls due to lack of confirmed positive samples for SANGV IgG.

Antigen slides were prepared from transfected cells fixed on 12 well plates. The cells were harvested by detaching cells using trypsin and centrifugation and resuspended using PBS/FBS solution to allow cells to attach on the slides. The cells were fixed on the slides using cold acetone and washed with PBS. Human serum samples as well as controls from commercial kits were applied and incubated at 37^0C. An FITC conjugated goat antihuman secondary antibody was applied and 4/176 samples reacted with the recombinant NP of SANGV. With each preparation of NP in transiently transfected cells, the amount of antigen varies from slide to slide and hence, a positive control was run each time to avoid the possibility of false negative results.

Attempts at an in-house ELISA were performed using diluted NP antigen at 1:10; 1:140; 1:160 and 1:320. Undiluted antigen and mock antigen was used to optimize the assay. Positive and negative control human serum from commercial ELISA kits were used to test for antibodies that cross-react with the African NP antigen. Two human serum samples that were positive by IFA using the recombinant NP of SANGV were tested for reactivity in ELISA. The resulting high OD values are suggestive of a non-specific reaction between controls and cell preparation. To reduce non-specific reaction, the recombinant NP was diluted and there was still no differentiation between the net OD values for positive and negative. This is indicative of low concentrations of recombinant NP antigen for reactivity. Mammalian expression yields low concentrations of protein and is a problem when preparing sufficient antigen for ELISA.

Overall, there were four African hantavirus reactors by IFA, six Eurasian reactors and three American reactors by ELISA. There were no samples that reacted against all three antigens. Reactors were strongly positive. Additional borderline reactors were noted. The lack of concordance between the reactors, although difficult to fully explain, could be a result of heterogeneity between the antigens. In phylogenetic studies, SANGV shares a node and common ancestor with European and Asian hantaviruses including DOBV, HNTV and SEOV with distance estimation of 0,6 to 0,7 in the nucleotide sequence and 0,2 in the amino acids sequences respectively (Klempa et al., 2006). Among hantaviruses that group together genetically, hantaviruses from the same geographical area have a higher homology which is expected as they share reservoir hosts and are genetically more closely related (Appendix D).

Chapter 4: Discussion

Hantaviruses belong to the *Bunyaviridae* family and have a negative sense tri-segmented genome. Hantaviruses belong to the genus *Hantavirus* that was formally defined in 1985 .The genome consists of the small, medium and large (S, M and L) RNA gene segments encoding the NP, glycoproteins and RdRp respectively (Hart and Bennet., 1999; Hooper et al., 2001; Jaaskelainen et al., 2007; Meisel et al., 2006; Plyusnin et al., 1996; Prescott et al., 2005). Based on geographic distribution, hantaviruses are divided into old and new world viruses. Old world viruses include hantaviruses in Europe and Asia and new world viruses are found in the Americas. Within each group, there exists a great divergence and viruses are further subdivided based on their nucleotide and amino acid sequences (Plyusnin & Morzunov, 2001; Jonsson et al, 2010). Hantaviruses are a global public health threat as they are the causative agents of a significant number of human illness with case reports of HFRS in China being approximately 20,000-50,000 per annum accounting for 90% of the total number of cases reported worldwide and an estimated 300 cases annually for HPCS in America with mortality rates as high as 60% (Cao et al., 2011; Muranyi et al., 2005; Jonsson et al., 2010; Zhang et al., 2010).

Humans, as well as rodents, are infected primarily by inhalation of aerosolized rodent excreta containing infectious virus (Easterbrook & Klein, 2008; Vaheri et al, 2008). Behavioural patterns of the persistently infected reservoir hosts greatly affect the distribution of a hantavirus in a population. Aggressive behaviour found in males which results in wounds is responsible for transmission of hantaviruses and the most wounded animals would more likely be infected (Escutanaire et al, 2002; Hinson et al, 2004). Humans have also been infected through contact with open wounds and less frequently through rodent bites (Hammerbeck et al, 2008).

Upon the discovery of HNTV (Lee et all., 1978), the search for hantaviruses intensified and the distribution of existing viruses expanded. Since its discovery, HNTV has been detected in Korea, China, Mongolia, and eastern Russia. Another HFRS causing virus, Seoul, and other related hantaviruses have been identified in most parts of Asia, Europe and the Americas due to the spread of the reservoir hosts which are members of the *Rattus* genus which have a worldwide distribution (Mackow & Gavrilovskaya, 2001; Jonsson et al., 2010; Lee., 1996).

Disease association with African hantaviruses including SANGV has not been found, but a potential for these, and other unidentified hantaviruses in Africa, to cause disease in

humans is likely. There is a possibility that disease caused by hantaviruses in Africa has been undetected or misdiagnosed due to other diseases such as rickettsial diseases, severe pneumonia, leptospirosis and other viral haemorrhagic fevers found in Africa with similar symptoms (Bi et al, 2008). It was not until 1984 that some data on hantavirus infections in Africa was available through serological testing (Gonzalez et al., 1984). Human and rodent sera from Central and West African countries including Benin, Burkino Faso, the Central African Republic and Gabon were tested using IFA and Vero E6 cells infected with HNTV strain 76-118. A total of 1718 human sera and 176 rodent sera were collected between 1980-84 and tested by IFA for the presence of Hantaan specific antibodies. A seroprevalence of 1,8% and 9,2% for human and rodents respectively was reported. From the 31 positive human sera, half were positive at a 1:64 or higher dilution considered to indicate a response highly specific for Hantaan-related viruses. However, only four of these 15 samples were positive by PRNT with the 76-118 strain. The absence of neutralising ability indicates a lack of a homologous virus strain from Africa and therefore warranted further studies (Gonzalez et al., 1984). A survey was conducted in the following years from 1985-87 in Cameroon, Central African Republic, Chad, Congo, Equatorial Guinea and Gabon (Gonzalez et al., 1989). Using Hantaan virus infected Vero cells fixed on slides, a cohort of 5070 randomly selected people were tested for hantavirus specific antibodies by IFA. The survey reported a prevalence of 6.15% (Gonzalez et al, 1989). Subsequently human and rodent sera from Senegal, Nigeria, Djibouti, and Egypt were found to be positive for hantavirus infection by IFA and ELISA using HNTV and SEOV antigens. A seroprevalence of 16,6% and 11,5% was reported for human and rodent sera respectively. For Nigeria, a seroprevalence of 0.2-1.2% was reported for human sera. Human and rodent sera in Djibouti showed a 3,3% and 5,2% prevalence respectively (Tomori et al, 1986; Rodier et al., 1993; Saluzzo et al, 1985). A serosurvey of children aged between 8-14 years was conducted in 1989 in Egypt to test for hantavirus antibody prevalence among others and reported a seroprevalence of 9% using ELISA (Corwin et al, 1992).

Prior to the discovery of SANGV in 2006, South African rodent and human sera had been surveyed for antibodies against hantaviruses. A seroprevalence of 2,3% was detected in 221 rodents screened by IFA using HNTV, SEOV, PUUV and PHV antigens. Confirmatory assays involved the use of differential IFA and PRNT (Lee et al, 1999). Using the same antigens from Asia and Europe origin, a seroprevalence of 0,9% and 1,2% in 1418 human patients with fever and renal failure was also reported (Lee et al., 1999).

SANGV was the first African hantavirus that was identified in 2006 using a pan-hanta RT-PCR. The assay targets the conserved regions of the L segment. Hantaviral nucleic acid was amplified from a blood specimen of an African wood mouse and virus was later isolated in cell culture (Klempa et al, 2006, 2012). SANGV was found to be closely related to the European hantavirus, DOBV, through phylogenetic studies of the nucleotide and amino acid sequences of all three viral segments and groups together with Murinae hantaviruses such as SEOV and HNTV. Phylogenetic analysis of the NP of DOBV and SANGV showed 96 % identity (Klempa et al, 2006; Hettonen et al, 2008).

A study was conducted from 2010 to 2012 for hantavirus detection and identification in small mammals such as rodents and shrews. Samples included over 2000 lung specimens from three muridae species and insectivore species representing the *Crocidura* and *Myosorex* genera as well as the genus *Elephantulus*. Rodents were captured in the Western and Eastern Cape province, KwaZulu Natal, Northern Cape, Gauteng and Namibia. The pan-hanta RT- PCR was used targeting the L segment. No positive samples were detected in the tested rodents and the study was reported to be ongoing. For the human samples, a cohort of 1442 patients samples from the Western Cape province were screened initially using recombinant NP of DOBV and PUUV in IgG ELISA and a 14,5% seroprevalence was reported. Confirmatory test including Western blot and IFA assay using NP of DOBV and PUUV antigens were used as well as PRNT and IgM ELISA. The final seroprevalence of 1,0% in humans in South Africa was reported. This is as a result of the sensitivity and specificity of the confirmatory tests used. An additional ten samples from patients at Groote Schuur and Tygerberg Hospitals in the Western Cape presenting with fever, acute renal failure or acute hepatitis and/or rash were tested. Seven out of ten were positive but no confirmatory tests could be performed (Witkowski et al., 2014).

Serological cross reactivity among European and American hantavirus antigens are well documented (Elgh et al., 1998; Vilibić-Čavlek et al., 2015; Lindkvist et al.,1997; Sjolander et al., 1999; Guzman et al., 2013; McKenzie et al., 1999; Salonen et al., 1998;Tischler et al., 2008). Cross-reactivity varies to a certain degree and is often observed in acute sera using PUUV, HNTV, SNV, SEOV and Saareema virus antigens. NP based ELISA revealed an IgG serotype cross-reactivity targeting the amino-terminal region of NP which increases after onset of illness (Elgh et al.,1998).

However, results from serological surveys show that despite detection of antibodies using European, Asian and American antigens, a lower seroprevalence was confirmed using neutralization assays. This suggests the presence of African specific hantaviruses with some serological cross reactivity but the degree of cross reactivity is not clear. Hence, the development of African hantavirus recombinant antigen for application in serological studies is warranted. Of the ten hantaviruses identified in Africa, the NP gene sequence is available for six on GenBank and three, SANGV, Kilimanjaro virus and Bowé virus, have complete NP gene sequences. Pairwise nucleotide and amino acid sequence identities between the S, M and L gene segments of HNTV, SEOV, DOBV, PUUV, Tula virus, SNV, ANDV, TPMV, Imjin virus and Rockport virus revealed a nucleotide sequence identities of the S segment encoding the NP to be 52,3% to 74,5% and amino acid identities of 44,0% to 86,0% (Plyusnin and Morzunov, 2001).

The aim of the study was to prepare a recombinant African hantavirus antigen for the development of serological in-house assays. The complete ORF of the S gene of Sangassou strain SA14 was retrieved from an online data tool. The nucleotide sequence was codon optimized. In codon optimization, rare codons in the gene are changed to favour those used by the host without changing the amino acid sequence encoding for the protein (Gustafsson et al., 2004). A DNA or protein sequence and a table or reference set of the host codon usage are required for optimization (Puigbo et al., 2007).

CAI and effective number of codons (Enc) are two ways in which codon bias can be evaluated (Sharp and Li, 1987; Wright, 1990). The similarity between the codon usage of the gene and a reference group is measured by the CAI. The values range from 0 to 1,1 being same codon usage. It is the most commonly used and most effective measure of codon bias in predicting gene expression level (Lithwick and Margalit, 2003; Henry and Sharp, 2007; Sharp and Li, 1987).

The codon optimized gene was modified by the addition of a Hisx6 tag at the C-terminus and this was done for purification and for easy detection of the NP using anti-histidine antibodies. In addition, the Kozak sequence was inserted before the start codon for enhanced expression of the protein. The gene was cloned into the mammalian expression vector pcDNA 3.1(+) using the EcoRI and XbaI sites that are present on the vector and the construct was designated SANGV_pcDNA3.1(+). The plasmid DNA construct was synthesized and supplied. Transient expression of the recombinant antigen in transfected cells was

confirmed by IFA using monoclonal mouse anti-histidine to detect the histidine tag and SDS-PAGE and Western blot assay.

The most common laboratory method used for detection of antibodies against hantaviruses is IFA. One of the first serological assays as reviewed for diagnosis of hantaviruses in Europe and Asia, was the IFA (Machado et al., 2009) Traditionally, hantavirus infected cells fixed on glass plates were used as antigens. The disadvantage of using hantavirus infected cells or working directly with virus is the need for a BSL 3 or 4 laboratory and the viruses were difficult to culture with low virus yields reported. All three of the structural proteins of hantaviruses; NP, Gc and Gn can be produced through recombinant techniques and be used in IFA. The NP is most commonly used because of its abundance and strong humoral immune response (Kallio-Kokko et al., 2001). Bacterial and mammalian systems are the most commonly used for expression of proteins. Based on application of protein, the choice to use either depends on the yield and whether the need exists for a protein that has undergone post-translational modification for functionality. Bacterial systems are often used for maximal protein yield but the product is a protein that does not go through phosphorylation, glycosylation, precursor processing and targeting (Miller, 1988; Luckow 1991; OReilly et al., 1994). In a previous study in our laboratory a CCHF NP, expressed using a bacterial system was unstable and hence in this study a mammalian expression system was used.

Nucleotide sequence of the NP of SANGV SA14 and the codon optimized NP gene were aligned. The encoding amino acids of the codon optimized gene corresponds with the native SA14 gene. For confirmation, the optimized gene was translated and aligned using Clustal Omega with the translated SA14 gene and showed 100% aa identity. An approximately 1393bp band was observed on a 1% agarose gel and the band corresponds with the size of the S gene of SANGV by estimation using a DNA ladder of 100 to 1000 bp products. After sequencing, data was edited and aligned with the codon optimized gene and this was performed to confirm that the supplied construct had the S gene of SANGV. The ratio of 2:0,75 was chosen as optimal for expression of NP in BHK-21 cells and the same was decided for the positive control GFP when cells were transfected with lipofectamine reagent. An SDS-PAGE gel analysis and Western blot assay revealed a 50 kDa protein that corresponds to the NP of hantaviruses.

Antigen slides were prepared for in-house IFA. Transfected cells were harvested and fixed on 12 well slides. The expression of a recombinant SANGV NP was initially confirmed by detection of the histidine tag using IFA on transiently transfected cells. The expression of a biologically functional protein, in other words recombinant NP that reacted with hantavirus antibodies, was confirmed using control sera provided in commercial kits. Positive human control sera from kits with antigens from Europe, Asia and America reacted with the NP depending on the kit, from batch to lot number, to confirm biologically active NP due to a lack of a SANGV positive control in our laboratory. Based on reactivity, one positive control was chosen for use in-house IFA.

Serum samples from patients with acute illness and fever with suspected tick bite fever were screened for antibodies against hantaviruses by IFA using cells transiently transfected with a SANGV and by commercial ELISA. Although positive reactors were identified, there was no concordance between the results. Two commercial kits, one for detection of hantavirus antibodies against HTNV, DOBV and PUUV recombinant NP antigens and one for antibodies against ANDV and SNV were used to screen the samples. Published serological surveys from geographically distinct regions have consistently shown that despite heterologous cross reactivity using IFA and ELISA, results using neutralisation assays are usually non concordant with a lower rate of seropositive samples (Okumura et al., 2007; De Padua et al., 2015; Ogino et al., 2003). This indicates that heterologous hantaviruses do not cross react in neutralisation assays. It also suggests either some degree of non specific serological cross reactivity is common or that there are different hantaviruses circulating with differences in cross reactivity. Either way the application of an African hantavirus in serological surveys of rodents would certainly contribute to trying to identify if there are hantaviruses circulating in other regions of Africa. In the absence of neutralisation assays for African hantaviruses, due to inherent difficulties in culturing some hantaviruses, serological tools play an important role in epidemiological studies.

In this study, we report a serological tool that can be used to detect the presence of hantaviruses in southern Africa. The usefulness of transiently transfected cells in producing a biologically functional recombinant SANGV NP has been employed but with the need to increase yields in order to develop an in-house ELISA. Future studies aim to improve yield of recombinant NP by the development of a stable cell line. This may help to increase the yield of protein for ELISA. A serological survey using the NP of SANGV can also be performed in future to screen rodents captured in the Free State and human sera from other at risk

populations for antibodies against hantaviruses. Due to serological cross reactivity of the NP of hantaviruses, perhaps recombinant NP antigens from Europe, Asia and America can be prepared and used with the recombinant NP of SANGV in serological assays. Furthermore, to investigate the serological cross-reactivity between the hantaviruses, a useful approach would be to immunise mice with each recombinant antigen and assess the degree of specific cross reactivity. Although the results of the serological survey in this study are inconclusive, this is similar to many previous studies where results are inconclusive and nonconcordant when using heterologous antigens. This may also suggest that SANGV, while present in West Africa, may not be the representative hantavirus for southern Africa. The presence of multiple known reservoir species, and the results of sero-surveys suggesting antibody positive reactors, warrants further investigation into the presence of hantaviruses in southern Africa.

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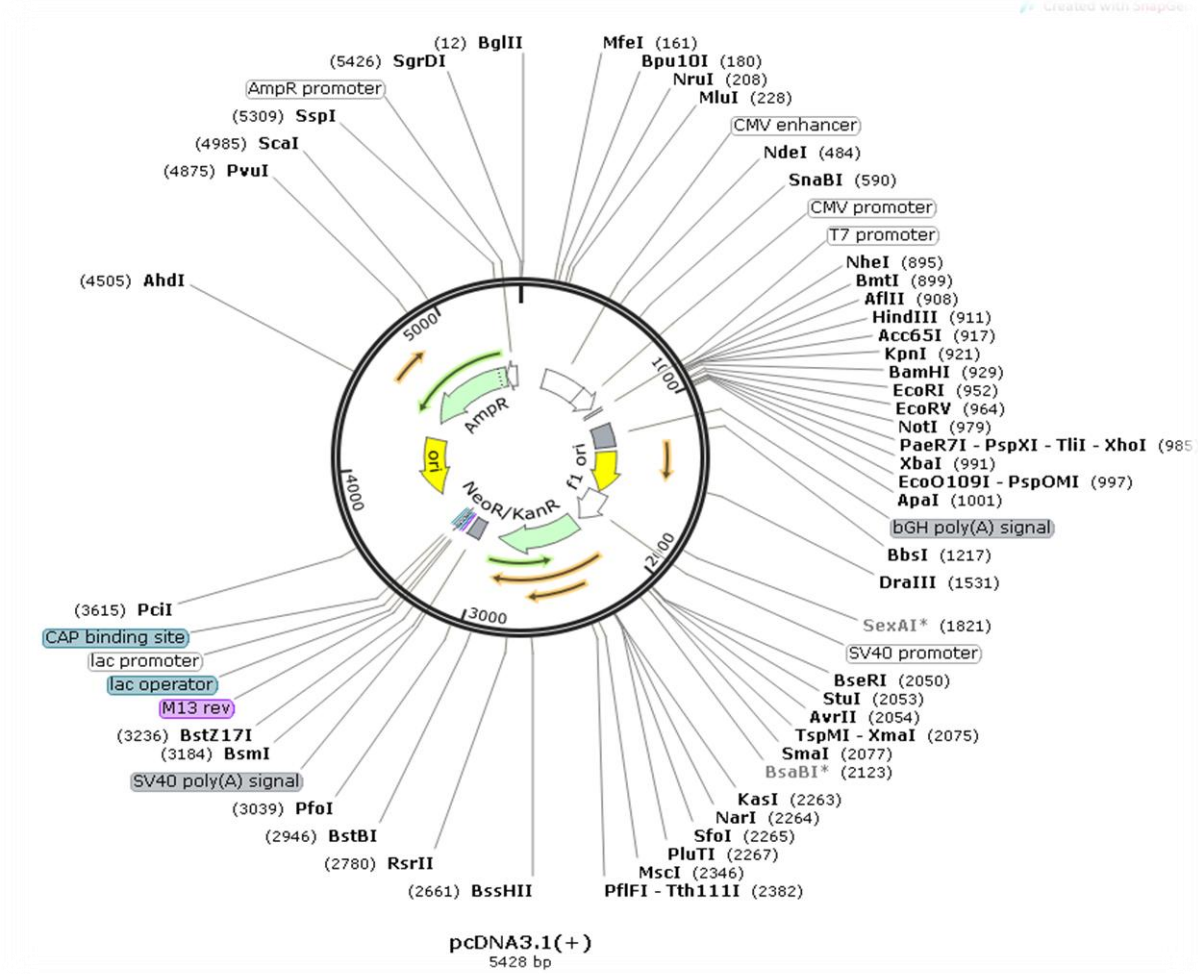
Appendices

Appendix A: Plasmid vector map



A1: Mammalian expression vector, pcDNA3.1 (+). The vector consist of important elements such as the T7 and BGH priming sites, a CMV promoter, Ampicillin resistance gene and the Neomycin resistance gene. The recombinant Sangassou gene was cloned in the EcoRI and XbaI restriction sites. The gene was constructed to include the Kozak sequence and the His tag.

Appendix B: Media composition, buffers and solutions

SOC medium

20g Bacto-tryptone
5g Bacto-yeast extract
2ml 5M NaCl
2. 5ml 1M KCl
10ml 1M MgSO4
20ml 1M Glucose
(Dissolved in 900ml distilled water and autoclaved)
Stored at 4°C.

1xTBS
Dissolve 6,05 g Tris and 8,76 g NaCl in 800 ml deionised water. Adjust pH to 7,5 using 1 M HCl, adjust volume to 1 L using deionised water and store at 4°C.

1% Agarose gel
1g Agarose
100ml 1X TAE buffer
6µl 10mg/ml Ethidium bromide

50× TAE buffer
242ml Tris base in 500ml distilled water
57.1ml Glacial acetic acid
100ml 0.5M EDTA (pH 8)
Adjust with water to 1L
1×TAE prepared by diluting 1:50

IFA Blocking solution
50L 1X PBS
5g saccharose
250µl Triton x-100

IFA wash solution
1ml TWEEN® 20 detergent (Cal Biochem, Darmstadt, Germany)
100ml 1×PBS

0.1% PBS solution
2µl TWEEN® 20 detergent (Cal Biochem, Darmstadt, Germany)
2 L 1x PBS

LB (amp or kan) broth
5g Yeast extract
10g Tryptone
10g NaCl
Dissolved in 1L distilled water
100ug/ml Ampicillin or 10µg/ml Kanamycin (filter sterilized)

LB/amp or kan plates

2g Yeast extract
5g Tryptone
5g NaCl
7.5g Agar
Dissolved in 500ml distilled water
100ug/ml Ampicillin or 10µg/ml Kanamycin(filter sterilized)

0,2% Coomassie Brilliant Blue stain (1 L)
Dissolve 1 g Coomassie Brilliant Blue in 450 ml methanol and 100 ml glacial acetic acid. Adjust volume to 1 L using deionised water and store at room temperature**.**

Destaining solution (2 L)
Mix 600 ml methanol, 200 ml glacial acetic acid and 1,2 L deionised water and store at room temperature.

Transfer buffer (1 L)
Dissolve 2,9 g glycine, 5,8 g Tris and 0,37 g SDS in 200 ml methanol. Adjust volume to 1 L using deionised water and store at 4°C.

BSA buffer
Dissolve 4,3g NaCl (MW=58,44g/mol), 1,04g $Na_2EDTA:2H_2O$ (MW=372,24g/mol) and 3,02g tris (hydroxymethyl) aminomethane (MW121.14g/mol) in 500 ml d H_2O. Add 2,5ml Nonidet P40 and mix thoroughly. Store at room temperature. Supplement an aliquot of lysis buffer with 0,1% bovine serum albumin before use.

Appendix C: Raw ELISA data (Commercial antihantavirus pool 1&2 kits)

OD values at 450nm. Positive sera with antihantavirus antibodies are highlighted in yellow after calculating the ratio from OD values. Results highlighted in blue show serum sample readings that were borderline.

Plate 1: Anti-hantavirus "pool 1" Eurasia. Serum samples C1-10/10/26

	1	2	3	4	5	6	7	8	9	10
A	0,1240	0,0540	0,0870	0,2110	0,0440	0,0480	0,0840	0,0460	0,0170	0,0560
B	0,0000	0,1730	0,0830	0,1270	0,0890	0,0180	0,6450	0,1490	0,4000	0,0570
C	0,0410	0,2560	0,0660	0,0620	0,1470	0,0330	0,1130	0,0260	0,0480	0,0250
D	0,3840	0,1570	0,1380	0,0980	0,0450	0,0290	0,1120	0,0680	0,1400	0,0480
E	0,0280	0,0090	0,0760	0,3200	0,0260	0,1610	0,1860	0,0380	0,0690	0,0480
F	0,0000	0,1320	0,0310	0,0860	0,0470	0,0480	0,0470	0,0580	0,0450	0,0850
G	0,1020	0,2340	0,1300	0,0980	0,0110	0,0230	0,0940	0,0130	0,0220	0,0280
H	0,0400	0,0000	0,0200	0,0420	0,0220	0,0700	0,1010	0,0390	0,0340	0,1810

	1	2	3	4	5	6	7	8	9	10
A	0,41196	0,179402	0,289037	0,700997	0,146179	0,159468	0,27907	0,152824	0,056478	0,186047
B	0	0,574751	0,275748	0,421927	0,295681	0,059801	2,142857	0,495017	1,328904	0,189369
C	0,136213	0,850498	0,219269	0,20598	0,488372	0,109635	0,375415	0,086379	0,159468	0,083056
D	1,275748	0,521595	0,458472	0,325581	0,149502	0,096346	0,372093	0,225914	0,465116	0,159468
E	0,093023	0,0299	0,252492	1,063123	0,086379	0,534884	0,61794	0,126246	0,229236	0,159468
F	0	0,438538	0,10299	0,285714	0,156146	0,159468	0,156146	0,192691	0,149502	0,282392
G	0,33887	0,777409	0,431894	0,325581	0,036545	0,076412	0,312292	0,043189	0,07309	0,093023
H	0,13289	0	0,066445	0,139535	0,07309	0,232558	0,335548	0,129568	0,112957	0,601329

Plate 2: Anti-hantavirus “pool 2” America. Serum samples C1-10/10/26

	1	2	3	4	5	6	7	8	9
A	0,0530	0,0250	0,0620	0,1410	0,0360	0,0320	0,0250	0,0190	0,0120
B	0	0,0760	0,0270	0,0810	0,0250	0,0150	0,1290	0,0330	0,0680
C	0,0120	0,1610	0,0270	0,0160	0,0260	0,0140	0,0420	0,0180	0,0670
D	0,0840	0,0400	0,0490	0,0280	0,0620	0,0160	0,0750	0,0510	0,0750
E	0,0320	0,0150	0,0530	0,1680	0,0120	0,0550	0,0550	0,0110	0,0850
F	0,0880	0,1000	0,0600	0,0210	0,0170	0,0360	0,0140	0,0230	0,0200
G	0,0000	0,0980	0,0380	0,0460	0,0430	0,0130	0,0360	0,0070	0,0180
H	0,0000	0,0540	0,0170	0,0160	0,0150	0,1280	0,0790	0,0200	0,0200

	1	2	3	4	5	6	7	8	9
A	0,247664	0,116822	0,28972	0,658879	0,168224	0,149533	0,116822	0,088785	0,056075
B	0	0,35514	0,126168	0,378505	0,116822	0,070093	0,602804	0,154206	0,317757
C	0,056075	0,752336	0,126168	0,074766	0,121495	0,065421	0,196262	0,084112	0,313084
D	0,392523	0,186916	0,228972	0,130841	0,28972	0,074766	0,350467	0,238318	0,350467
E	0,149533	0,070093	0,247664	0,785047	0,056075	0,257009	0,257009	0,051402	0,397196
F	0,411215	0,46729	0,280374	0,098131	0,079439	0,168224	0,065421	0,107477	0,093458
G	0	0,457944	0,17757	0,214953	0,200935	0,060748	0,168224	0,03271	0,084112
H	0	0,252336	0,079439	0,074766	0,070093	0,598131	0,369159	0,093458	0,093458

Plate 1: Anti-hantavirus "pool 1" Eurasia. Serum samples 10/10/25-60/9/3

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	0,3010	0,7000	0,1610	0,0830	0,3180	0,1010	0,1000	0,2120	0,2830	0,0400	0,0370	0,1020
B	0,7100	0,0330	0,1200	0,0890	0,0360	0,1190	0,0320	0,0540	0,1460	0,0790	0,0280	0,0330
C	0,0410	0,2140	0,0630	0,1400	0,0690	0,1000	0,0950	0,0660	0,0880	0,1070	0,1200	0,0930
D	0,1380	0,0540	0,1320	0,0820	0,0560	0,0580	0,0120	0,0250	0,1880	0,0270	0,0150	0,0000
E	0,0580	0,0390	0,0310	0,0090	0,0500	0,0440	0,0210	0,0530	0,0120	0,0840	0,0710	0,0000
F	0,0400	0,0210	0,1720	0,0130	0,0390	0,1560	0,0510	0,0470	0,0270	0,0720	0,0360	0,0000
G	0,1750	0,2060	0,0810	0,0960	0,0250	0,0080	0,1070	0,1160	0,2440	0,3060	0,0760	0,0000
H	0,2680	0,1020	0,1490	0,1460	0,0080	0,0330	0,0310	0,0840	0,0450	0,0430	0,1250	0,0000

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2,325581	0,534884	0,275748	1,056478	0,335548	0,332226	0,704319	0,940199	0,13289	0,122924	0,33887
B	2,358804	0,109635	0,398671	0,295681	0,119601	0,395349	0,106312	0,179402	0,48505	0,262458	0,093023	0,109635
C	0,136213	0,710963	0,209302	0,465116	0,229236	0,332226	0,315615	0,219269	0,292359	0,355482	0,398671	0,30897
D	0,458472	0,179402	0,438538	0,272425	0,186047	0,192691	0,039867	0,083056	0,624585	0,089701	0,049834	0
E	0,192691	0,129568	0,10299	0,0299	0,166113	0,146179	0,069767	0,17608	0,039867	0,27907	0,23588	0
F	0,13289	0,069767	0,571429	0,043189	0,129568	0,518272	0,169435	0,156146	0,089701	0,239203	0,119601	0
G	0,581395	0,684385	0,269103	0,318937	0,083056	0,026578	0,355482	0,385382	0,810631	1,016611	0,252492	0
H	0,890365	0,33887	0,495017	0,48505	0,026578	0,109635	0,10299	0,27907	0,149502	0,142857	0,415282	0

Plate 2: Anti-hantavirus "pool 2". Serum samples 10/10/25-60/9/3

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,2140	0,8920	0,0860	0,0340	0,0370	0,0460	0,0230	0,0620	0,0540	0,0140	0,0150	0,0460
B	0,8340	0,0190	0,0630	0,0300	0,0180	0,1290	0,0180	0,0420	0,0230	0,0240	0,0140	0,0250
C	0,0180	0,0750	0,0530	0,0860	0,0150	0,0290	0,0810	0,0520	0,0440	0,3050	0,0620	0,0630
D	0,0520	0,0200	0,0480	0,0390	0,0170	0,0290	0,0100	0,0110	0,0260	0,0180	0,0100	0,0000
E	0,0330	0,0280	0,0120	0,0080	0,0180	0,0200	0,0160	0,0320	0,0160	0,0110	0,0210	0,0000
F	0,0190	0,0110	0,0250	0,0190	0,0170	0,1020	0,0440	0,0620	0,0120	0,0210	0,0110	0,0000
G	0,0710	0,0900	0,0410	0,0330	0,0160	0,0120	0,0390	0,0400	0,0230	0,0690	0,0250	0,0000
H	0,2300	0,0560	0,0880	0,0830	0,0140	0,0330	0,0240	0,0250	0,0200	0,0150	0,0240	0,0000

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	4,168224	0,401869	0,158879	0,172897	0,214953	0,107477	0,28972	0,252336	0,065421	0,070093	0,214953
B	3,897196	0,088785	0,294393	0,140187	0,084112	0,602804	0,084112	0,196262	0,107477	0,11215	0,065421	0,116822
C	0,084112	0,350467	0,247664	0,401869	0,070093	0,135514	0,378505	0,242991	0,205607	1,425234	0,28972	0,294393
D	0,242991	0,093458	0,224299	0,182243	0,079439	0,135514	0,046729	0,051402	0,121495	0,084112	0,046729	0
E	0,154206	0,130841	0,056075	0,037383	0,084112	0,093458	0,074766	0,149533	0,074766	0,051402	0,098131	0
F	0,088785	0,051402	0,116822	0,088785	0,079439	0,476636	0,205607	0,28972	0,056075	0,098131	0,051402	0
G	0,331776	0,420561	0,191589	0,154206	0,074766	0,056075	0,182243	0,186916	0,107477	0,32243	0,116822	0
H	1,074766	0,261682	0,411215	0,38785	0,065421	0,154206	0,11215	0,116822	0,093458	0,070093	0,11215	0

Calculation of ELISA results

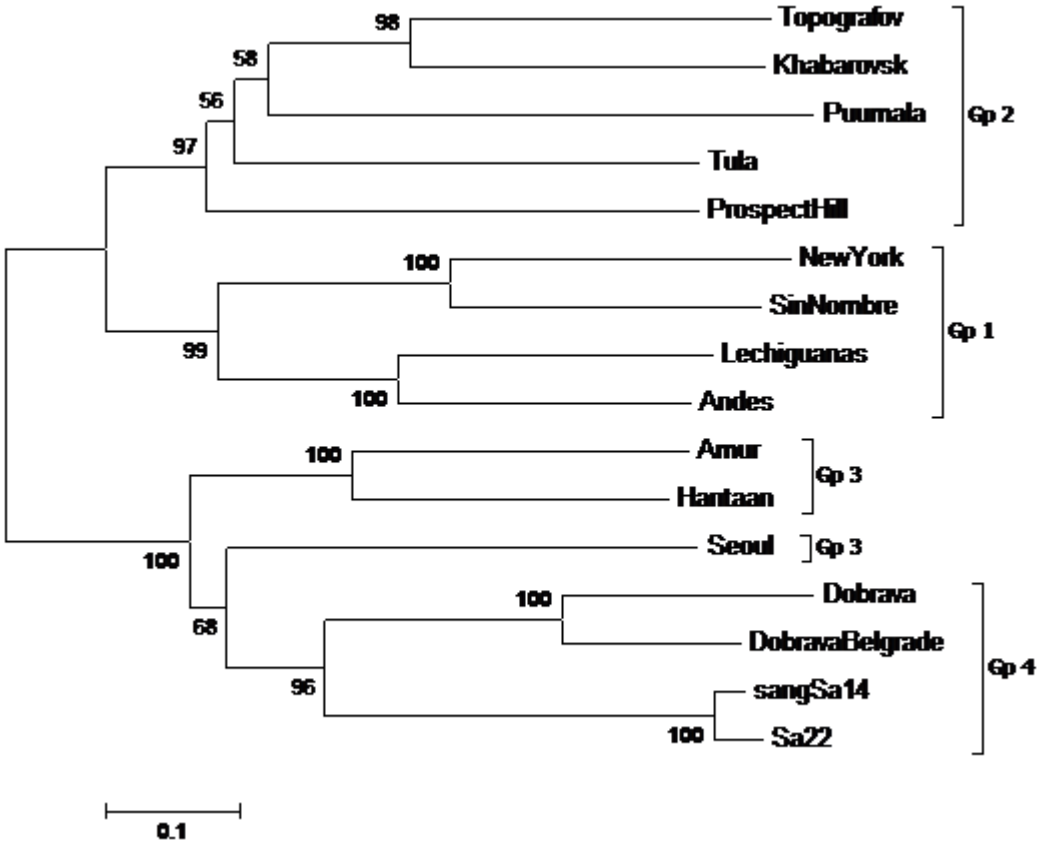
Ratio = Extinction of the control or patient sample/ Extinction of calibrator 2

Interpretation of results:

Ratio <0.8: negative
Ratio ≥0.8 to <1.1: borderline
Ratio ≥1.1: positive

Appendix D: Phylogenetic tree

The genetic relationship of hantaviruses was determined using sequence data for the complete NP of selected hantaviruses retrieved from GenBank. Sequence data available on request.



Phylogenetic tree of hantavirus based on the NP nucleotide sequences. A common ancestor is shared for SANGV strains and the European hantaviruses including Amur, HNTV, SEOV, and close relation between DOBV strain viruses is observed as compared to American and some European hantaviruses. The estimates of average evolutionary divergence over sequence pairs within groups (Gp) were 0,25; 0,213; 0,166; and 0,263 for Gp 1, 3, 4 and 2 respectively. The analysis involved 16 nucleotide sequences. Evolutionary analyses were conducted in MEGA6

Appendix E: Abstract: Presentation, Faculty Forum, August 2016, Faculty of Health Science, University of the Free State.

KR -1

Title: **PREPARATION OF RECOMBINANT ANTIGEN FOR SEROLOGICAL DETECTION OF AFRICAN HANTAVIRUSES**

Authors: D Damane, FJ Burt

Departments: Medical Microbiology and Virology

Presenter: Deborah Damane

Introduction: Hantaviruses are rodent-borne viruses belonging to the Bunyaviridae family, genus Hantavirus. The first African hantavirus was identified in 2006 and subsequently nine more have been identified. The emergence of hantaviruses has public health implications. The presence of hantaviruses in southern Africa has not been confirmed. Serological studies showed a 1% seroprevalence in human sera from the Western Cape using antigen prepared from European hantaviruses. The aim of the study was to investigate serological assays for the detection of antibody against African hantaviruses using a recombinant nucleoprotein (rNP) of Sangassou virus (SANGV).

Method: The open reading frame (ORF) of the nucleocapsid protein (NP) of Sangassou virus was retrieved from GenBank. The sequence was codon optimized for expression in a mammalian system and the gene was modified to include a histidine tag at the 3'end and a kozak sequence at the 5' end. Restriction sites were also included in the gene to facilitate cloning. Baby hamster kidney (BHK) cells were transfected using the construct. Transfected cells were fixed on antigen slides for screening human serum samples for IgG antibody against hantaviruses.

Results: The NP gene of Sangassou virus was synthesized and supplied in expression plasmid, pcDNA3.1(+). The sequence of the plasmid DNA was confirmed by sequencing. Expression of the recombinant NP was confirmed using Western blot analysis and immunofluorescence by detection of the histidine tag.

Conclusion: The r NP is a safe reagent for developing serological assays and will be used to screen human sera for IgG antibodies against hantaviruses.

Appendix F: Sequencing data (SANG_pcDNA3.1 (+))

ATGGCCACCCTGGAGGAGATCCAGAAGGAGATCAACATCCACGAGGGCCAGCTGATCATCGCCAAGCAGAA
GGTGAAGGACGCCGAGAAGCAGTACGAGAAGGACCCCGACGACCTGAACAAGAGGGCCCTGAGCGACAGG
GAGGGCATCGCCCACAGCATCCAGAGCAAGATCGACGAGCTGAGGAGGCAGCTGGCCGACAGGGTGGCCGC
CGGCAGGAACCTGGGCCAGGAGAGGGACCCCACCGGCGTGGAGCCCGGCGACCACCTGAGGGAGAAGAGC
ATGCTGAGCTACGGCAACGTGATCGACCTGAACCACCTGGACATCGACGAGCCCACCGGCCAGACCGCCGAC
TGGCTGAGCATCGTGATCTACCTGACCAGCTTCGTGGTGCCCATCCTGCTGAAGGCCCTGTACATGCTGACCA
CCAGGGGCAGGCAGACCACCAAGGACAACAAGGGCATGAGGATCAGGTTCAAGGACGACAGCAGCTTCGAG
GACGTGAACGGCATCAGGAAGCCCAAGCACCTGTTCCTGAGCATGCCCAACGCCCAGAGCAGCATGAAGGCC
GAGGAGATCACCCCCGGCAGGTTCAGGACCGCCGTGTGCGGCCTGTACCCCGCCCAGATCAAGGCCAGGAAC
ATGGTGAGCCCCGTGATGAGCGTGATCGGCTTCATCACCCTGGCCAGGGACTGGACCGAGAGGATCGAGAA
CTGGCTGGACCAGCCCTGCAAGTTCATGAGCGAGCCCAGCCAGACCAGCCTGCAGAAGGGCCCCGCCACCAA
CAGGGACTACCTGAACCAGAGGCAGGCCAGCCTGGCCCAGATGGAGACCAAGGAGGCCCAGGCCGTGAGGC
AGCAGGCCGTGGACGCCGGCTGCAACCTGGTGGACCACATCGACAGCCCCAGCAGCATCTGGGTGTTCGCCG
GCGCCCCCGACAGGTGCCCCCCCACCTGCCTGTTCATCAGCGGCATGGCCGAGCTGGGCGCCTTCTTCAGCAT
CCTGCAGGACATGAGGAACACCATCATGGCCAGCAAGACCGTGGGCACCAGCGAGGAGAAGCTGAGGAAGA
AGAGCAGCTTCTACCAGAGCTACCTGAGGAGGACCCAGAGCATGGGCATCCAGCTGGACCAGAGGATCATCA
TCATGTTCATGGTGGAGTGGGGCAAGGAGGCCGTGGACGGCTTCCACCTGGGCGACGACATGGACCCCGAG
CTGAGGAGCTTCGCCCAGGCCCTGATCGACCAGAAGGTGAAGGAGATCAGCAACCAGGAGCCCCTGAAGAT
CCACCACCACCACCACCACTAAT

Appendix G: Nucleotide and amino acid sequence alignment of the codon optimized S gene of SANGV and the native SANGV SA14 S gene. The gene was modified by addition of the Kozak sequence and the histidine tag highlighted in blue, stop codons in red and the EcoRI and XbaI restriction sites.

G	**A**	**A**	**T**	**T**	**C**	**G**	**C**	**C**	**G**	**C**	**C**	**A**	**C**	**C**
EcoRI site						**Kozak sequence**								

Nucleotide sequence of codon optimized NP gene
Nucleotide sequence of SA14 NP gene
Encoded amino acid
Description

A	**T**	**G**	**G**	**C**	**C**	**A**	**C**	**C**	**C**	**T**	**G**	**G**	**A**	**G**	**G**	**A**	**G**	**A**	**T**	**C**	**C**	**A**	**G**	**A**	**A**	**G**	**G**	**A**	**G**
A	T	G	G	C	A	A	C	A	C	T	A	G	A	G	G	A	G	A	T	C	C	A	A	A	A	G	G	A	A
Met			Ala			Thr			Leu			Glu			Glu			Ile			Gln			Lys			Glu		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

A	**T**	**C**	**A**	**A**	**C**	**A**	**T**	**C**	**C**	**A**	**C**	**G**	**A**	**G**	**G**	**G**	**C**	**C**	**A**	**G**	**C**	**T**	**G**	**A**	**T**	**C**	**A**	**T**	**C**
A	T	C	A	A	C	A	T	C	C	A	T	G	A	G	G	G	T	C	A	A	C	T	A	A	T	T	A	T	A
Ile			Asn			Ile			His			Glu			Gly			Gln			Leu			Ile			Ile		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

G	**C**	**C**	**A**	**A**	**G**	**C**	**A**	**G**	**A**	**A**	**G**	**G**	**T**	**G**	**A**	**A**	**G**	**G**	**A**	**C**	**G**	**C**	**C**	**G**	**A**	**G**	**A**	**A**	**G**
G	C	A	A	A	G	C	A	A	A	A	G	G	T	G	A	A	G	G	A	T	G	C	T	G	A	A	A	A	A
Ala			Lys			Gln			Lys			Val			Lys			Asp			Ala			Glu			Lys		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

C	**A**	**G**	**T**	**A**	**C**	**G**	**A**	**G**	**A**	**A**	**G**	**G**	**A**	**C**	**C**	**C**	**C**	**G**	**A**	**C**	**G**	**A**	**C**	**C**	**T**	**G**	**A**	**A**	**C**
C	A	G	T	A	T	G	A	G	A	A	G	G	A	T	C	C	T	G	A	T	G	A	C	C	T	T	A	A	T
Gln			Tyr			Glu			Lys			Asp			Pro			Asp			Asp			Leu			Asn		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

A	**A**	**G**	**A**	**G**	**G**	**G**	**C**	**C**	**C**	**T**	**G**	**A**	**G**	**C**	**G**	**A**	**C**	**A**	**G**	**G**	**G**	**A**	**G**	**G**	**G**	**C**	**A**	**T**	**C**
A	A	G	A	G	G	G	C	A	C	T	C	A	G	T	G	A	T	A	G	A	G	A	A	G	G	A	A	T	T
Lys			Arg			Ala			Leu			Ser			Asp			Arg			Glu			Gly			Ile		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

G	**C**	**C**	**C**	**A**	**C**	**A**	**G**	**C**	**A**	**T**	**C**	**C**	**A**	**G**	**A**	**G**	**C**	**A**	**A**	**G**	**A**	**T**	**C**	**G**	**A**	**C**	**G**	**A**	**G**
G	C	T	C	A	T	T	C	C	A	T	C	C	A	G	A	G	C	A	A	A	A	T	A	G	A	T	G	A	G
Ala			His			Ser			Ile			Gln			Ser			Lys			Ile			Asp			Glu		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

C	**T**	**G**	**A**	**G**	**G**	**A**	**G**	**G**	**C**	**A**	**G**	**C**	**T**	**G**	**G**	**C**	**C**	**G**	**A**	**C**	**A**	**G**	**G**	**G**	**T**	**G**	**G**	**C**	**C**
T	T	G	C	G	G	A	G	A	C	A	A	C	T	T	G	C	T	G	A	T	A	G	G	G	T	T	G	C	A
Leu			Arg			Arg			Gln			Leu			Ala			Asp			Arg			Val			Ala		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

G	**C**	**C**	**G**	**G**	**C**	**A**	**G**	**G**	**A**	**A**	**C**	**C**	**T**	**G**	**G**	**G**	**C**	**C**	**A**	**G**	**G**	**A**	**G**	**A**	**G**	**G**	**G**	**A**	**C**
G	C	A	G	G	A	C	G	G	A	A	C	C	T	G	G	G	G	C	A	A	G	A	A	C	G	A	G	A	T
Ala			Gly			Arg			Asn			Leu			Gly			Gln			Glu			Arg			Asp		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

C	**C**	**C**	**A**	**C**	**C**	**G**	**G**	**C**	**G**	**T**	**G**	**G**	**A**	**G**	**C**	**C**	**C**	**G**	**G**	**C**	**G**	**A**	**C**	**C**	**A**	**C**	**C**	**T**	**G**
C	C	A	A	C	C	G	G	T	G	T	T	G	A	A	C	C	A	G	G	A	G	A	T	C	A	T	C	T	C
Pro			Thr			Gly			Val			Glu			Pro			Gly			Asp			His			Leu		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

A	**G**	**G**	**G**	**A**	**G**	**A**	**A**	**G**	**A**	**G**	**C**	**A**	**T**	**G**	**C**	**T**	**G**	**A**	**G**	**C**	**T**	**A**	**C**	**G**	**G**	**C**	**A**	**A**	**C**
A	G	A	G	A	A	A	A	A	T	C	C	A	T	G	C	T	C	A	G	T	T	A	T	G	G	A	A	A	T
Arg			Glu			Lys			Ser			Met			Leu			Ser			Tyr			Gly			Asn		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

G	**T**	**G**	**A**	**T**	**C**	**G**	**A**	**C**	**C**	**T**	**G**	**A**	**A**	**C**	**C**	**A**	**C**	**C**	**T**	**G**	**G**	**A**	**C**	**A**	**T**	**C**	**G**	**A**	**C**
G	T	T	A	T	C	G	A	C	T	T	G	A	A	C	C	A	T	C	T	A	G	A	T	A	T	T	G	A	T
Val			Ile			Asp			Leu			Asn			His			Leu			Asp			Ile			Asp		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

G	**A**	**G**	**C**	**C**	**C**	**A**	**C**	**C**	**G**	**G**	**C**	**C**	**A**	**G**	**A**	**C**	**C**	**G**	**C**	**C**	**G**	**A**	**C**	**T**	**G**	**G**	**C**	**T**	**G**
G	A	A	C	C	A	A	C	T	G	G	G	C	A	G	A	C	T	G	C	A	G	A	C	T	G	G	C	T	G
Glu			Pro			Thr			Gly			Gln			Thr			Ala			Asp			Trp			Leu		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

A	**G**	**C**	**A**	**T**	**C**	**G**	**T**	**G**	**A**	**T**	**C**	**T**	**A**	**C**	**C**	**T**	**G**	**A**	**C**	**C**	**A**	**G**	**C**	**T**	**T**	**C**	**G**	**T**	**G**
A	G	T	A	T	T	G	T	G	A	T	T	T	A	C	T	T	G	A	C	T	T	C	A	T	T	T	G	T	G
Ser			Ile			Val			Ile			Tyr			Leu			Thr			Ser			Phe			Val		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

G	T	G	C	C	C	A	T	C	C	T	G	C	T	G	A	A	G	G	C	C	C	T	G	T	A	C	A	T	G
G	T	G	C	C	A	A	T	T	T	T	G	T	T	G	A	A	G	G	C	A	C	T	T	T	A	T	A	T	G
Val			Pro			Ile			Leu			Leu			Lys			Ala			Leu			Tyr			Met		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

C	T	G	A	C	C	A	C	C	A	G	G	G	G	C	A	G	G	C	A	G	A	C	C	A	C	C	A	A	G
C	T	T	A	C	A	A	C	A	A	G	A	G	G	G	A	G	G	C	A	A	A	C	T	A	C	T	A	A	G
Leu			Thr			Thr			Arg			Gly			Arg			Gln			Thr			Thr			Lys		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

G	A	C	A	A	C	A	A	G	G	G	C	A	T	G	A	G	G	A	T	C	A	G	G	T	T	C	A	A	G
G	A	T	A	A	C	A	A	G	G	G	A	A	T	G	A	G	G	A	T	T	A	G	G	T	T	T	A	A	G
Asp			Asn			Lys			Gly			Met			Arg			Ile			Arg			Phe			Lys		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

G	A	C	G	A	C	A	G	C	A	G	C	T	T	C	G	A	G	G	A	C	G	T	G	A	A	C	G	G	C
G	A	C	G	A	C	A	G	C	T	C	T	T	T	T	G	A	A	G	A	T	G	T	C	A	A	T	G	G	G
Asp			Asp			Ser			Ser			Phe			Glu			Asp			Val			Asn			Gly		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

A	T	C	A	G	G	A	A	G	C	C	C	A	A	G	C	A	C	C	T	G	T	T	C	C	T	G	A	G	C
A	T	T	A	G	A	A	A	G	C	C	T	A	A	G	C	A	T	C	T	G	T	T	T	T	T	A	T	C	G
Ile			Arg			Lys			Pro			Lys			His			Leu			Phe			Leu			Ser		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

A	**T**	**G**	**C**	**C**	**C**	**A**	**A**	**C**	**G**	**C**	**C**	**C**	**A**	**G**	**A**	**G**	**C**	**A**	**G**	**C**	**A**	**T**	**G**	**A**	**A**	**G**	**G**	**C**	**C**
A	T	G	C	C	A	A	A	T	G	C	A	C	A	A	T	C	T	A	G	T	A	T	G	A	A	G	G	C	T
Met			Pro			Asn			Ala			Gln			Ser			Ser			Met			Lys			Ala		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

G	**A**	**G**	**G**	**A**	**G**	**A**	**T**	**C**	**A**	**C**	**C**	**C**	**C**	**C**	**G**	**G**	**C**	**A**	**G**	**G**	**T**	**T**	**C**	**A**	**G**	**G**	**A**	**C**	**C**
G	A	A	G	A	A	A	T	T	A	C	T	C	C	A	G	G	A	A	G	A	T	T	C	C	G	C	A	C	T
Glu			Glu			Ile			Thr			Pro			Gly			Arg			Phe			Arg			Thr		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

G	**C**	**C**	**G**	**T**	**G**	**T**	**G**	**C**	**G**	**G**	**C**	**C**	**T**	**G**	**T**	**A**	**C**	**C**	**C**	**C**	**G**	**C**	**C**	**C**	**A**	**G**	**A**	**T**	**C**
G	C	A	G	T	T	T	G	T	G	G	A	T	T	G	T	A	T	C	C	A	G	C	A	C	A	G	A	T	A
Ala			Val			Cys			Gly			Leu			Tyr			Pro			Ala			Gln			Ile		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

A	**A**	**G**	**G**	**C**	**C**	**A**	**G**	**G**	**A**	**A**	**C**	**A**	**T**	**G**	**G**	**T**	**G**	**A**	**G**	**C**	**C**	**C**	**C**	**G**	**T**	**G**	**A**	**T**	**G**
A	A	A	G	C	A	A	G	A	A	A	T	A	T	G	G	T	C	A	G	C	C	C	A	G	T	G	A	T	G
Lys			Ala			Arg			Asn			Met			Val			Ser			Pro			Val			Met		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

A	**G**	**C**	**G**	**T**	**G**	**A**	**T**	**C**	**G**	**G**	**C**	**T**	**T**	**C**	**A**	**T**	**C**	**A**	**C**	**C**	**C**	**T**	**G**	**G**	**C**	**C**	**A**	**G**	**G**
A	G	T	G	T	T	A	T	T	G	G	C	T	T	T	A	T	T	A	C	T	C	T	A	G	C	C	A	G	A
Ser			Val			Ile			Gly			Phe			Ile			Thr			Leu			Ala			Arg		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

G	**A**	**C**	**T**	**G**	**G**	**A**	**C**	**C**	**G**	**A**	**G**	**A**	**G**	**G**	**A**	**T**	**C**	**G**	**A**	**G**	**A**	**A**	**C**	**T**	**G**	**G**	**C**	**T**	**G**
G	A	C	T	G	G	A	C	T	G	A	A	A	G	A	A	T	T	G	A	A	A	A	T	T	G	G	C	T	T
Asp			Trp			Thr			Glu			Arg			Ile			Glu			Asn			Trp			Leu		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

G	**A**	**C**	**C**	**A**	**G**	**C**	**C**	**C**	**T**	**G**	**C**	**A**	**A**	**G**	**T**	**T**	**C**	**A**	**T**	**G**	**A**	**G**	**C**	**G**	**A**	**G**	**C**	**C**	**C**
G	A	C	C	A	A	C	C	T	T	G	C	A	A	A	T	T	T	A	T	G	T	C	T	G	A	A	C	C	A
Asp			Gln			Pro			Cys			Lys			Phe			Met			Ser			Glu			Pro		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

A	**G**	**C**	**C**	**A**	**G**	**A**	**C**	**C**	**A**	**G**	**C**	**C**	**T**	**G**	**C**	**A**	**G**	**A**	**A**	**G**	**G**	**G**	**C**	**C**	**C**	**C**	**G**	**C**	**C**
T	C	T	C	A	G	A	C	T	T	C	A	T	T	G	C	A	G	A	A	G	G	G	C	C	C	T	G	C	A
Ser			Gln			Thr			Ser			Leu			Gln			Lys			Gly			Pro			Ala		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

A	**C**	**C**	**A**	**A**	**C**	**A**	**G**	**G**	**G**	**A**	**C**	**T**	**A**	**C**	**C**	**T**	**G**	**A**	**A**	**C**	**C**	**A**	**G**	**A**	**G**	**G**	**C**	**A**	**G**
A	C	A	A	A	T	A	G	A	G	A	C	T	A	T	C	T	A	A	A	C	C	A	A	A	G	G	C	A	G
Thr			Asn			Arg			Asp			Tyr			Leu			Asn			Gln			Arg			Gln		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

G	**C**	**C**	**A**	**G**	**C**	**C**	**T**	**G**	**G**	**C**	**C**	**C**	**A**	**G**	**A**	**T**	**G**	**G**	**A**	**G**	**A**	**C**	**C**	**A**	**A**	**G**	**G**	**A**	**G**
G	C	C	T	C	C	C	T	T	G	C	A	C	A	A	A	T	G	G	A	A	A	C	A	A	A	A	G	A	A
Ala			Ser			Leu			Ala			Gln			Met			Glu			Thr			Lys			Glu		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

G	**C**	**C**	**C**	**A**	**G**	**G**	**C**	**C**	**G**	**T**	**G**	**A**	**G**	**G**	**C**	**A**	**G**	**C**	**A**	**G**	**G**	**C**	**C**	**G**	**T**	**G**	**G**	**A**	**C**
G	C	T	C	A	G	G	C	T	G	T	C	A	G	A	C	A	A	C	A	A	G	C	A	G	T	T	G	A	T
Ala			Gln			Ala			Val			Arg			Gln			Gln			Ala			Val			Asp		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

G	**C**	**C**	**G**	**G**	**C**	**T**	**G**	**C**	**A**	**A**	**C**	**C**	**T**	**G**	**G**	**T**	**G**	**G**	**A**	**C**	**C**	**A**	**C**	**A**	**T**	**C**	**G**	**A**	**C**
G	C	T	G	G	T	T	G	T	A	A	T	T	T	A	G	T	G	G	A	C	C	A	C	A	T	T	G	A	C
Ala			Gly			Cys			Asn			Leu			Val			Asp			His			Ile			Asp		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

A	**G**	**C**	**C**	**C**	**C**	**A**	**G**	**C**	**A**	**G**	**C**	**A**	**T**	**C**	**T**	**G**	**G**	**G**	**T**	**G**	**T**	**T**	**C**	**G**	**C**	**C**	**G**	**G**	**C**
T	C	A	C	C	A	T	C	A	T	C	A	A	T	T	T	G	G	G	T	A	T	T	T	G	C	A	G	G	A
Ser			Pro			Ser			Ser			Ile			Trp			Val			Phe			Ala			Gly		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

G	**C**	**C**	**C**	**C**	**C**	**G**	**A**	**C**	**A**	**G**	**G**	**T**	**G**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**A**	**C**	**C**	**T**	**G**	**C**	**C**	**T**	**G**
G	C	A	C	C	T	G	A	T	C	G	C	T	G	T	C	C	A	C	C	A	A	C	C	T	G	C	C	T	G
Ala			Pro			Asp			Arg			Cys			Pro			Pro			Thr			Cys			Leu		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

T	**T**	**C**	**A**	**T**	**C**	**A**	**G**	**C**	**G**	**G**	**C**	**A**	**T**	**G**	**G**	**C**	**C**	**G**	**A**	**G**	**C**	**T**	**G**	**G**	**G**	**C**	**G**	**C**	**C**
T	T	C	A	T	A	T	C	A	G	G	T	A	T	G	G	C	T	G	A	A	C	T	A	G	G	A	G	C	T
Phe			Ile			Ser			Gly			Met			Ala			Glu			Leu			Gly			Ala		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

T	**T**	**C**	**T**	**T**	**C**	**A**	**G**	**C**	**A**	**T**	**C**	**C**	**T**	**G**	**C**	**A**	**G**	**G**	**A**	**C**	**A**	**T**	**G**	**A**	**G**	**G**	**A**	**A**	**C**
T	T	C	T	T	T	T	C	A	A	T	A	C	T	G	C	A	G	G	A	T	A	T	G	A	G	A	A	A	T
Phe			Phe			Ser			Ile			Leu			Gln			Asp			Met			Arg			Asn		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

A	**C**	**C**	**A**	**T**	**C**	**A**	**T**	**G**	**G**	**C**	**C**	**A**	**G**	**C**	**A**	**A**	**G**	**A**	**C**	**C**	**G**	**T**	**G**	**G**	**G**	**C**	**A**	**C**	**C**
A	C	A	A	T	T	A	T	G	G	C	A	T	C	A	A	A	A	A	C	T	G	T	C	G	G	G	A	C	A
Thr			Ile			Met			Ala			Ser			Lys			Thr			Val			Gly			Thr		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

A	**G**	**C**	**G**	**A**	**G**	**G**	**A**	**G**	**A**	**A**	**G**	**C**	**T**	**G**	**A**	**G**	**G**	**A**	**A**	**G**	**A**	**A**	**G**	**A**	**G**	**C**	**A**	**G**	**C**
T	C	T	G	A	G	G	A	A	A	A	G	C	T	T	A	G	G	A	A	G	A	A	A	T	C	G	T	C	A
Ser			Glu			Glu			Lys			Leu			Arg			Lys			Lys			Ser			Ser		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

T	**T**	**C**	**T**	**A**	**C**	**C**	**A**	**G**	**A**	**G**	**C**	**T**	**A**	**C**	**C**	**T**	**G**	**A**	**G**	**G**	**A**	**G**	**G**	**A**	**C**	**C**	**C**	**A**	**G**
T	T	C	T	A	C	C	A	G	T	C	T	T	A	C	C	T	T	A	G	A	A	G	A	A	C	G	C	A	A
Phe			Tyr			Gln			Ser			Tyr			Leu			Arg			Arg			Thr			Gln		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

A	**G**	**C**	**A**	**T**	**G**	**G**	**G**	**C**	**A**	**T**	**C**	**C**	**A**	**G**	**C**	**T**	**G**	**G**	**A**	**C**	**C**	**A**	**G**	**A**	**G**	**G**	**A**	**T**	**C**
T	C	T	A	T	G	G	G	A	A	T	A	C	A	A	C	T	T	G	A	T	C	A	G	A	G	G	A	T	A
Ser			Met			Gly			Ile			Gln			Leu			Asp			Gln			Arg			Ile		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

A	**T**	**C**	**A**	**T**	**C**	**A**	**T**	**G**	**T**	**T**	**C**	**A**	**T**	**G**	**G**	**T**	**G**	**G**	**A**	**G**	**T**	**G**	**G**	**G**	**G**	**C**	**A**	**A**	**G**
A	T	T	A	T	C	A	T	G	T	T	C	A	T	G	G	T	G	G	A	A	T	G	G	G	G	T	A	A	A
Ile			Ile			Met			Phe			Met			Val			Glu			Trp			Gly			Lys		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

G	**A**	**G**	**G**	**C**	**C**	**G**	**T**	**G**	**G**	**A**	**C**	**G**	**G**	**C**	**T**	**T**	**C**	**C**	**A**	**C**	**C**	**T**	**G**	**G**	**G**	**C**	**G**	**A**	**C**
G	A	G	G	C	T	G	T	T	G	A	T	G	G	A	T	T	T	C	A	T	C	T	T	G	G	T	G	A	T
Glu			Ala			Val			Asp			Gly			Phe			His			Leu			Gly			Asp		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

G	**A**	**C**	**A**	**T**	**G**	**G**	**A**	**C**	**C**	**C**	**C**	**G**	**A**	**G**	**C**	**T**	**G**	**A**	**G**	**G**	**A**	**G**	**C**	**T**	**T**	**C**	**G**	**C**	**C**
G	A	T	A	T	G	G	A	T	C	C	T	G	A	A	C	T	G	C	G	A	T	C	A	T	T	T	G	C	T
Asp			Met			Asp			Pro			Glu			Leu			Arg			Ser			Phe			Ala		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

C	**A**	**G**	**G**	**C**	**C**	**C**	**T**	**G**	**A**	**T**	**C**	**G**	**A**	**C**	**C**	**A**	**G**	**A**	**A**	**G**	**G**	**T**	**G**	**A**	**A**	**G**	**G**	**A**	**G**
C	A	G	G	C	A	T	T	G	A	T	T	G	A	T	C	A	G	A	A	A	G	T	G	A	A	A	G	A	G
Gln			Ala			Leu			Ile			Asp			Gln			Lys			Val			Lys			Glu		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

A	**T**	**C**	**A**	**G**	**C**	**A**	**A**	**C**	**C**	**A**	**G**	**G**	**A**	**G**	**C**	**C**	**C**	**C**	**T**	**G**	**A**	**A**	**G**	**A**	**T**	**C**	**C**	**A**	**C**
A	T	A	T	C	T	A	A	T	C	A	G	G	A	A	C	C	A	C	T	T	A	A	G	A	T	T			
Ile			Ser			Asn			Gln			Glu			Pro			Leu			Lys			Ile			His		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																													

C	**A**	**C**	**C**	**A**	**C**	**C**	**A**	**C**	**C**	**A**	**C**	**C**	**A**	**C**	**T**	**A**	**A**	**T**	**C**	**T**	**A**	**G**	**A**
															T	A	G						
His			His			His			His			His			Stop			Ser			Arg		
Codon Optimized SANGV NP gene/ Sangassou SA14 NP gene																		**XbaI site**					

Appendix H: Permission to use figure

Our Ref: LA/IMBY/P9765

17 February 2017

Dear Deborah Damane,

Material requested: 1 Figure – Fig1 from Dionysios Christos Watson, Maria Sargianou, Anna Papa, Paraskevi Chra, Ioannis Starakis & George Panos (2014) Epidemiology of Hantavirus infections in humans: A comprehensive, global overview, Critical Reviews in Microbiology, 40:3, 261-272

Thank you for your correspondence requesting permission to reproduce the above mentioned material from our Journal in your printed thesis entitled 'Preparation of recombinant antigen for serological detection of African hantaviruses' and to be posted in your university's repository - University of the Free State.

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Yours sincerely

Lee-Ann

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Web: www.tandfonline.com
e-mail: lee-ann.anderson@tandf.co.uk

Appendix I: Ethics approval

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

IRB nr 00006240
REC Reference nr 230408-011
IORG0005187
FWA00012784

26 February 2016

DR DR DAMANE
DEPT OF MEDICAL MICROBIOLOGY AND VIROLOGY
UFS

Dear Dr Damane

ECUFS 118/09B
PROJECT TITLE: PREPARATION OF RECOMBINANT ANTIGEN FOR SEROLOGICAL DETECTION OF AFRICAN HANTA VIRUSES.

1. You are hereby kindly informed that, at the meeting held on 23 February 2016, the Health Sciences Research Ethics Committee (HSREC) approved the above project.

2. The Committee must be informed of any serious adverse event and/or termination of the study.

3. Any amendment, extension or other modifications to the protocol must be submitted to the HSREC for approval.

4. A progress report should be submitted within one year of approval and annually for long term studies.

5. A final report should be submitted at the completion of the study.

6. Kindly use the **HSREC NR** as reference in correspondence to the HSREC Secretariat.

7. The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

Yours faithfully

.....
DR SM LE GRANGE
CHAIR: HEALTH SCIENCES RESEARCH ETHICS COMMITTEE

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | F: +27 (0)51 444 4359 | E: ethicsfhs@ufs.ac.za
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agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/4

Prof F J Burt
Department of Medical Microbiology and Virology
Faculty of Health Sciences
University of the Free State

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Dear Prof Burt

Your fax / memo / letter/ E-mail dated 25 May 2015, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study, except the BHK cell line for which a dispensation is attached. Chemically synthesized Hantavirus may only be stored if permission is obtained in terms of the Non-proliferation of Weapons of Mass Destruction Act, 1993 (Act No 87 of 1993);
3. Only chemically synthesized , non-infectious Hantavirus may be used during the study;
4. No wild rodents or any other animals may be screened during this study;
5. Approval may be needed in terms of the Genetically Modified Organisms Act, 1997 (Act No 15 of 1997);
6. A veterinary import permit must be obtained prior to the importation of the BHK cell line;
7. Solid Waste Technologies SA (Pty) Ltd must be used as accredited waste management company.

Title of research/study: Preparation of recombinant antigens for serological surveillance
Researcher (s): Prof F J Burt
Institution: Department of Medical Microbiology and Virology, Faculty of Health Sciences, University of Free State
Your Ref./ Project Number:
Our ref Number: 12/11/1/4

Kind regards,

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
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Reference: 12/11/1/4

Prof F J Burt
Department of Medical Microbiology and Virology
Faculty of Health Sciences
University of Free State

Dear Prof Burt,

RE: Dispensation on Section 20 Approval in Terms of the Animal Diseases Act, 1984 (Act No 35 of 1984) for: Preparation of recombinant antigens for serological surveillance

Your email dated 25 May 2015 refers.

A dispensation is hereby granted on point 2 of the Section 20 approval that was issued for the above mentioned study (attached):

i) ATCC BHK cell line may be stored at the Department of Medical Microbiology and Virology, Faculty of Health Sciences, University of Free State;
ii) Stored BHK cell line may only be used for further research after having obtained new Section 20 approval;
iii) Stored BHK cell line may not be outsourced.

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH