Humoral immune responses against novel recombinant replication-competent poxvirus candidate vaccines expressing full length and chimeric lyssavirus glycoprotein genes

by

Mutungi Evans

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Department of Microbiology and Plant Pathology
Faculty of Natural and Agricultural Science
University of Pretoria
Pretoria, South Africa

Supervisors: Prof Louis H. Nel
Co-supervisors: Dr Wanda Markotter
Dr Jacqueline Weyer

Declaration:

I, Mutungi Evans Mulandi declare that the thesis/dissertation, which I hereby submit for the degree MSc (Microbiology) at the University of Pretoria, is my own work and has not been previously submitted by me for a degree at this or any other tertiary institution.

SIGNATURE:……

DATE:……..

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List of abbreviations

µl	Microliter
aa	Amino acid
ABLV	Australian bat lyssavirus
BHK	Baby hamster kidney
BC	Before Christ
bp	Base pair
BLAST	Basic Local Alignment Search Tool
^{0}C	Celsius
CDC	Centres for Disease Control and Prevention, Atlanta, USA
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CTL	Cytolytic T-lymphocytes
CNS	Central nervous system
CVS	Challenge Virus Standard
DMEM/F12	Dulbecco's Modified Eagle's Medium and Ham's F12 medium
DNA	Deoxyribonucleic Acid
DUVV	Duvenhage virus
EBLV	European bat lyssavirus
E. coli	*Escherichia coli*
EEV	Extracellular enveloped virus
EGFP	Enhanced green fluorescence protein
ERA	Evelyn-Rockitnicki-Abelseth
FCS	Fetal calf serum
ffd_{50}	50 % Focus forming dose
FITC	Fluorescein isothiocyanate
gt	Genotype
gpt	Guanyl phosphoribosyl transferase
G	Glycoprotein
HDCV	Human diploid cell vaccine
IgG	Immunoglobulin

i.m	Intramuscular
IMV	Intracellular mature virus
IFN γ	Interferon γ
kb	Kilo base pair
KDa	Kilo Dalton
LBV	Lagos bat virus
MNA	Mouse neuroblastoma
m.o.i	Multiplicity of infection
MOKV	Mokola virus
mAbs	Monoclonal antibody
MHC I	Major histocompatibility complex class I
mRNA	Messenger ribonucleic acid
NICD	National Institute for Communicable Diseases of the National Health Laboratory Service
N	Nucleoprotein
NK	Natural killer
ORF	Open reading frame
PCECV	Purified chicken embryo cell vaccine
P	Phosphoprotein
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEP	Post-exposure prophylaxis
PFU	Plaque forming units
PV	Pasteur virus
RABV	Rabies virus
RFFIT	Rapid fluorescent focus inhibition test
RIG	Rabies immunoglobulin
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SHIBV	Shimoni bat virus
TK	Thymidine kinase

VNAbs	Virus neutralizing antibodies
V-RG	Vaccinia-rabies glycoprotein
WHO	World Health Organization

Summary

Rabies is a neurological disease caused by viruses of the genus *Lyssavirus* belonging to the family *Rhabdoviridae* and order *Mononegavirales.* The *Lyssavirus* genus consists of eleven species namely rabies virus; Lagos bat virus; Mokola virus , Duvenhage virus; European bat lyssavirus virus type 1; European bat lyssavirus type 2; Australian bat lyssavirus; Aravan; Khujand; Irkut; West Caucasian bat viruses and recently isolated Shimoni bat virus. The prototype virus of the *Lyssavirus* genus is represented by rabies virus. The remaining lyssaviruses are collectively denoted as rabies-related viruses. It has been proposed that these lyssavirus genotypes/species could be divided into 2 and possibly 3 phylogroups, based on diversity and shared biological/pathogenicity properties.

Currently available vaccines have proven efficacy against viruses that would make up phylogroup I, but fail to cross-protect against those lyssaviruses belonging to phylogroup II and III. The potential public health burden associated with mortality due to rabies-related virus infection has prompted several vaccine studies to focus on the pan-lyssavirus vaccine cross-protection. The studies demonstrated that lyssaviruses glycoprotein domains (antigenic sites) can be exchanged to generate chimeric vaccine constructs and that combining antigenic sites II and III of different lyssaviruses raised virus neutralizing antibodies against different lyssaviruses. The objective of our study was to determine the cross protective capacity of similar constructs in a vaccinia virus Copenhagen strain based recombinant in proof of concept to ascertain cross protection in a murine model. The current study substantiates findings of preceding studies that chimeric lyssavirus glycoprotein vaccine constructs conferred protection against homologous and heterologous lyssaviruses. The replicating vaccinia virus based vector demonstrated the benefit of a replicating recombinant vaccine vector as illustrated by the high protective neutralizing antibody titers obtained. The value of administering a booster dose was also highlighted in the higher antibody titers obtained upon a vaccine boost. The study clearly demonstrated that glycoprotein antigenic site II and III are not equal in inducing protection and that site II confers better protection against a homologous virus.

Chapter 1

1. LITERATURE REVIEW

1.1 Introduction

The disease rabies is caused by viruses of the *Lyssavirus* genus, in the *Rhabdoviridae* family. Rabies virus is the prototype virus in the *Lyssavirus* genus and the remaining lyssaviruses are collectively referred to as rabies-related viruses. Rabies is currently considered a neglected zoonotic disease (World Health Expert Consultation on Rabies, 2005) and remains prevalent in Africa and Asia where the problem is compounded by factors such as high incidence of immunodeficiency in resident populations, poverty, poor infrastructure and low levels of disease awareness. The disease has the highest case-fatality ratio of all diseases if post exposure prophylaxis is neglected (Hemachunda *et al*., 2002). Although rabies is preventable with immediate wound washing followed by administration of vaccines and immunoglobulin, the disease is certainly fatal upon onset of clinical symptoms. Moreover currently recommended commercial rabies biologics confer partial protection against some of these rabies-related lyssavirus (Hanlon *et al.,* 2005; Nel *et al.*, 2003). From this perspective, the investigation into efficacious and safe rabies vaccines that could also cross-protect against the rabies-related lyssaviruses endemic to the African subcontinent (Lagos bat and Mokola viruses), is a worthwhile undertaking.

In recent years research into rabies vaccinology has focused on enhancement of the cross-protective spectrum of rabies and rabies-related lyssaviruses (Jallet *et al.,* 1999; Bahloul *et al*., 1998; Nel *et al.,* 2003 and Weyer *et al*., 2007). It is thus imperative to investigate different vaccine systems that would improve cross-protection and induce a long lasting potent immune response. The recombinant vaccine vector vaccinia virus Copenhagen strain was chosen in this study.

1.1.1 History of rabies prophylaxis

Rabies is one of the oldest infectious diseases known to man dating back to the 23rd century before Christ (BC) as described in documents from ancient Mesopotamia. The word rabies has its origin in Sanskrit, 3000 years BC: "rabhas" meaning "do violence" (Steele and Fernandez, 1991). Nevertheless very little was known about rabies besides its association with saliva from rabid dog bites. It was not until 1880 when Louis Pasteur discovered the neurotropic nature of rabies and developed the first vaccine for rabies by serial passage of a rabies street virus in rabbit neuronal tissue (Pasteur, 1886). The nerve tissue vaccines were not without shortcomings, the vaccines caused vaccine-induced rabies in some of the vaccinated canines, and novel ways to reduce virulence of the rabies virus were explored. Subsequent modifications led to a breakthrough using potassium hydroxide to inactivate the virus and hence the first successful application of the Pasteur rabies vaccine, on 6 July 1885, the vaccine was administered to a severely bitten nine year old Joseph Meister who survived.

In years to follow various modifications were made on Pasteur's inactivated neural tissue vaccine mainly to improve its safety (reviewed in Wiktor, 1980; Wilkinson, 1988; Steele and Fernandez, 1991; Precausta and Soulebot, 1991). The approach used involved inactivation of the rabies virus with chemical agents (such as phenol and ether) or by physical means (such as ultraviolet radiation). Although phenol treated nerve tissue vaccines have been used for almost 70 years and continue to be used in some parts of Africa and Asia because there are cheap to produce (Dreesen, 1997), these vaccines have serious side effects due myelin induced encephalomyelitis (Javier *et al.,* 1989). Since the mid 19th century most developed countries have discontinued use of potentially harmful nerve tissue vaccines and began use of attenuated live rabies virus strains (reviewed in Bunn, 1991; Dreesen, 1997)(also see vaccine strains discussed in 1.2.1 and 1.2.2). The quest for safer rabies vaccines continues to explore further avenues, today developments in molecular science have produced much safer recombinant subunit vaccines, with the first recombinant vaccinia virus vaccine produced in 1982 (Mackett *et al*., 1984).

1.1.2 Immunoprophylaxis: the history and use of vaccines

Vaccination is the most valuable preventative measure in protecting individuals against infectious diseases. Several types of vaccines have been developed:

(a) Inactivated vaccines chemically treated with agents such as formaldehyde and beta-propiolactone or radiation of pathogenic virus such as the Pasteur's vaccine against rabies. The outer virion coat is left intact but DNA/RNA destroyed.

(b) Subunit vaccine comprising a viral component or antigen as part of virus antigen constructed into a plasmid recombined into viral vector genome.

(c) Attenuated live vaccines incorporating an avirulent virus made less pathogenic by cell culture passage or site directed mutagenesis.

The WHO recommends a number of fixed strains of RABV for vaccine production. These strains have an approved safety and antigenicity and include the Pasteur virus which is adapted to baby hamster kidney (BHK) cells, the Pitman Moore strain adapted to human diploid and Vero cells, Evelyn Rokitniki Abelseth (ERA) strain of Street Alabama Dufferin (SAD) virus adapted in BHK/21, Flury strain of RABV adapted in chicken embryo cells (CEC), Challenge virus strain (CVS) adapted to BHK-21 cells as well as its variant the Kissling strain (CVS-11). CVS is used as an international challenge virus in the detection of VNAbs to determine vaccine efficiency.

1.1.2.1 Attenuated vaccines

Oral vaccines have a benefit as vaccine immunogen because they can be used in conjunction with bait (Cross *et al*., 2006). Most vaccines used in oral vaccination of wild life have involved live virus attenuated by serial passage in cell culture such as the; Everlyn-Rockitnicki-Abelseth, strain of Street Alabama Dufferin virus and Vnukovo-32. The SAD Berne strain is a cell culture adapted strain of SAD and was used as a parent virus strain in developing SAD B19; SAD P5/88 and Avirulent-Gif Street Alabama Dufferin (SAG1) (WHO, 1998). SAD-B19 vaccine virus was used to orally vaccinate raccoons (*Procyon lotor*) and foxes (*Vulpes vulpes*). Although most of these vaccines have proved successful in wild life oral immunization, some limitations have been noted. The SAD-B19 caused vaccine-induced rabies in skunks (*Mephitis mephitis)* (Rupprecht *et al*., 1989). The SAG1 which contains a single mutation at arginine 333 in virus glycoprotein was considered genetically

unstable and has been replaced by SAG2. The SAG2 contains a double successive mutation at the same glycoprotein position which consequently reduced any residual pathogenicity (Cliquet *et al*., 2006). SAG-2 conferred improved genetic stability and is considered an effective vaccine in oral immunization of dogs, skunks and raccoons and has replaced SAG1 in vaccine production. SAG-2 vaccine is commercially available under the name Rabigen (Schumacher *et al*., 1993; Fekadu *et al*., 1996; Masson *et al*., 1996; Hanlon *et al*., 2002; Knobel *et al*., 2003).

1.1.2.2 Inactivated cell culture vaccines

In 1956 an embryonated tissue vaccine, the inactivated lyophilized duck embryo vaccine, was developed and was widely used until 1981. It was however discontinued due to a poor immune response in some vaccinees and also caused adverse reactions in rare cases. To minimize adverse reactions in human vaccinees the vaccine was propagated in human cell lines. In 1961, Hayflick and Moorhead developed a diploid human cell line, WI-38 (Hayflick and Moorhead, 1961). The Pitman-Moore strain of RABV was subsequently adapted to grow in WI-38 cell culture and a vaccine developed in collaboration with the Wistar Institute, Philadelphia, USA (Wiktor *et al*., 1964; Wiktor and Koprowski, 1965). The vaccine has become a gold standard for cell culture vaccines and is provided by Aventis Pasteur under the name *Imovax rabies*. It consists of the Pitman-Moore RABV strain grown on MRC-5 human diploid cell culture and concentrated by ultrafiltration, then inactivated in beta-propiolactone.

The WHO also recommends CEF and Vero cells for growing of cell culture vaccines (WHO, 2005). The PCEC vaccine provided by Chiron Vaccines under the name *Rabipur/RabAvert* is one such vaccine prepared from the fixed RABV Flury strain grown in CEF cultures (Scheiermann *et al*., 1987). Flury strain of RABV is passaged 136 times in one-day old chicks and then passaged a further two rounds in seven-day-old embryonated chicken eggs. The virus was further passaged and dubbed Flury Low-Egg-Passage (LEP) virus (40-50 passages) and High egg passage (HEP) (227-230 passages). The virus is inactivated in beta-propiolactone and concentrated by zonal centrifugation in a sucrose density gradient.
The rabies vaccine adsorbed is another vaccine prepared from the Kissling strain of CVS and adapted to fetal rhesus lung diploid cell culture. The vaccine is inactivated with beta-

propiolactone and concentrated by adsorption to aluminum phosphate. Rabies vaccines used for humans should meet certain requirements for the production and control of vaccines (WHO, 2007), if administered according to the WHO recommendations these vaccines provide complete protection against rabies (WHO, 2005).

1.1.2.3 Recombinant subunit vaccines

Improvement in rabies molecular biology has borne the next category of live vaccines involving live recombinant subunit vaccines (Dietzschold *et al.,* 1996). Recombinant vaccines generated through reverse genetics have been successfully used in Europe and United States of America to control rabies in wild life. A classical example is the virus rabies glycoprotein (V-RG) that has been successfully used in oral vaccination of wild life in Canada and Europe (Rupprecht *et al.,* 1988). This is a vaccinia virus recombinant expressing the ERA RABV strain glycoprotein. The rabies ERA strain cDNA was inserted in thymidine kinase (TK) locus of vaccinia virus Copenhagen strain (Kieny *et al*., 1984). DNA based vaccines have been used recently to study the cross protection of Lyssaviruses. Mice injected i.m with a eukaryotic expression vector carrying the glycoprotein-gene incorporated the viral DNA into host genome as confirmed by in vivo transcription and translation of the glycoprotein (Xiang *et al.,* 1996).

1.1.3 Pre- and post-exposure prophylaxis

Rabies is a fatal disease upon the onset of clinical symptoms, however it should be highlighted that with pre-exposure vaccination, this disease is preventable in humans and controllable in animals. The World Health Organization (WHO) recommendations for post exposure prophylaxis (PEP) provide for three categories; category 3 is high risk and involves single or multiple transdermal bites, scratches or contamination of mucous membranes with saliva through licks. In case of a category 3 exposure immediate washing and disinfection of the wound with ethanol or aqueous iodine solution followed by rapid administration of purified rabies immunoglobulin (RIG) and vaccine is recommended. Category 2 involves minor scratches without bleeding by a potentially rabid animal in which case only the vaccine is administered. Category 1 involves touching of suspected rabid animal and no treatment is given if animal exposure and/or vaccination history is reliable (Keates, 2010).

In the case of a category 3 and 2, wound cleaning is the first most important step. The rationale behind washing bite wounds is to physically remove and inactivate virus. The RIG provide immediate, passive, rabies virus neutralizing antibody (VNAb) coverage which persists for a short time (half-life of approximately 21 days) (Cabasso *et al*., 1971) but is sufficient until the patient responds to rabies vaccines by actively producing antibodies (WHO, 2002; Wilde *et al.,* 2002). There are two types of RIG are available, namely equine and human RIG, produced in vaccinated horses and hyper-immune human donors respectively. RIG is infiltrated into and around the wound site and any remaining solution should be injected at an intramuscular site distant from that of vaccine inoculation such as to the deltoid muscle (opposite to the deltoid muscle that received vaccine). The recommended dosage for RIG is: 20 IU/ kg for human RIG or 40 IU/ kg for equine RIG.

Two intramuscular vaccine regimens are generally used in pre- and post-exposure prophylaxis with tissue culture vaccines. The Essen scheme is the most widely used dose regimen (WHO, 2005), and consists of a single 1.0 ml dose of tissue culture vaccine given in the upper deltoid muscle in adults or anterolateral region of thigh in children on days 0, 3, 7, 14 and 28. Recently, due to limited vaccine supply and the costly implications of PEP, recommendations for rabies prevention and control in humans were revised. The Advisory Committee on Immunization Practices (ACIP) Rabies Workgroup (a subsidiary of ACIP) was formed to review rabies vaccine regimen options. An updated ACIP recommendation uses 4-1.0ml doses of vaccine regimen, replacing previously recommended 5-dose PEP regimen (ACIP: summary report, 2009). The 4 dose regimen was recently corroborated by the WHO (Keates, 2010). However this 4 dose schedule is recommended only in healthy, fully immunocompetent individuals (Rupprecht *et al*., 2009) and implications for its use within a population with a high level of immunocompromised individuals remains unknown. It is also important to point out that the ACIP is a structure whose recommendations are applicable only in the United States of America. The Zagreb scheme differs from the Essen scheme in that two 1.0 ml doses are administered in the upper deltoid muscles (one in each arm respectively) on day 0, with a single dose following on days 7 and 21 (WHO, 2005).

Intradermal vaccine schedules include the eight-site intradermal regimen and Thai Red

Cross intradermal regimen. The Thai Red Cross regimen ('2'2--22--22--00--11--11') consists of a dose of vaccine administered (0.1 ml in volume) intradermally at two lymphatic drainage sites, usually the left and right upper arm, on days 0, 3, 7 and 28. The vaccine should raise a visible bump ("bleb") on the skin. The purified Vero cell rabies vaccine (VerorabTM, ImovaxTM, Rabies vero TM, TRC Verorab TM) (Aventis Pasteur) and purified chick embryo cell vaccine (PCECV) (Rabipur™) (Chiron Vaccines) have been shown to be effective with this regimen (Beran *et al.,* 2005). This schedule is only useful where sufficient level of use of rabies vaccine is present in order to minimize vaccine wastage. This is because the rabies vaccines are packaged in 1.0ml doses for intramuscular use and not for intradermal use. Therefore vaccine would be wasted in a low usage setting where vaccine vials cannot be used up within the specified time (cost saving will be lost).

The eight-site intradermal regimen (8-0-4-0-1-1) consists of one dose of 0.1 ml vaccine administered intradermally at eight different sites, (usually upper arms, lateral thighs, suprascapular region, and lower quadrant of the abdomen) on day 0. Afterward, on day 7, four 0.1 ml injections are given intradermally into upper deltoid region of each arm as well as each lateral thigh. One additional dose is administered on days 28 and 90. The regimen is effective with human diploid cell vaccine (HDCV) (RabivacTM) and PCECV (Rabipur TM) vaccines regimen (WHO, 2005). Intradermal vaccination is used widely in South East Asia but not elsewhere. Several disadvantages include the difficulty of proper intradermal administration and the current format of vaccine distribution (1.0 ml vials) which will lead to vaccine wastage in low throughput settings.

Pre-exposure vaccination is mostly recommended to people at risk of exposure to lyssavirus infection, such as bat conservationists, veterinarians and laboratory researchers (WHO, 2002). The vaccine is administered in the same way as in post-exposure prophylaxis, but only three doses of vaccine are administered as opposed to five (or four). Persons who have previously completed the Essen vaccination regimen (pre-exposure or post exposure) with cell culture vaccine and previously had a documented rabies VNAb titer >0.5IU/ml should receive only 2 doses of vaccine: one on day 0 and the second on day 3 as PEP. Antibody titers of 0.5 IU/ml are recommended by the WHO as an indicator of an adequate adaptive immune response (WHO, 2005).

1.2 Etiology

Rabies is a neurological disease characterized by encephalitis. The etiological agents are lyssaviruses belonging to the family *Rhabdoviridae* and order *Mononegavirales*. The *Rhabdoviridae* family constitutes three genera that infect mammals, the *Lyssavirus*, *Vesiculovirus* and *Ephemerovirus.* The genus *Lyssavirus* was previously subdivided into seven genotypes (gts) based on antigenic traits and molecular sequencing of their genomes (Tordo *et al.,* 2006). In addition four putative lyssaviruses isolated from bat species in Eurasia namely Aravan (ARAV), Khujand (KHUV), Irkut (IRKV) and West Caucasian bat virus (WCBV) have been documented (Arai *et al*., 2003; Kuzmin *et al*., 2003 and Botvinkin *et al*., 2003). Recently a novel lyssavirus was discovered namely Shimoni bat virus (Kuzmin *et al*., 2010). The International Committee on Virus Taxonomy updated the lyssavirus taxonomy (ICTV Official Taxonomy: Updates since the 8th Report). Differentiation of genotypes has been based largely on genetic distances (identity values) between members of the genus, and on the bootstrap support of phylogenetic constructions. However, the ICTV does not operate with viral genotypes but rather recognizes only viral species. The updated taxonomy of the *Lyssavirus* genus now recognizes eleven species. These are namely rabies virus; Lagos bat virus; Mokola virus , Duvenhage virus; European bat lyssavirus virus-1; European bat lyssavirus-2, Australian bat lyssavirus, Aravan, Khujand, Irkut and West Caucasian bat virus. The Shimoni bat virus is still considered a putative specie. The species have originally been divided into two phylogroups based on genetic distances, serologic cross-reactivity, and peripheral pathogenicity in a mouse model. Rabies virus, Duvenhage virus, European bat lyssavirus virus-1 and 2 and Australian bat lyssavirus belong to phylogroups I while Lagos bat virus and Mokola virus belong to phylogroups II (Badrane *et al.,* 2001). Shimoni bat virus has been suggested to belong in phylogroups II of the lyssavirus genus. Although there is merit to the notion of the concept of lyssavirus phylogroups based on peripheral pathogenicity and other properties, details are still under discussion since the original proposal was based on single isolates for some of the species such as Lagos bat virus. For example, recent data on a larger panel of viruses showed that some Lagos bat virus isolates are much more pathogenic than others – in the same model and infection route (Markotter *et al*., 2009).

Recent analyses of the nucleoprotein (N), phosphoprotein (P) and glycoprotein (G) genes

have suggested the inclusion of the recently isolated new Eurasian bat lyssaviruses; Aravan, Khujand and Irkut viruses, into phylogroup I. However West Caucasian bat virus is most divergent of lyssaviruses and has a limited relatedness to Lagos bat virus and Mokola virus, and could form a separate phylogroup - phylogroup 3 (Kuzmin *et al*., 2005).

Currently available vaccines have proven efficacy against a subset of lyssaviruses referred to as phylogroup I and fail to cross protect against members of phylogroup II (figure 1.1) (and reviewed in Nel *et al.,* 2005). In a different study a varying partial protection was noted with the Eurasian lyssaviruses Aravan, Irkut and Khujand after pre-exposure with a commercial veterinary vaccine. However no protection was afforded against challenge with WCBV (Hanlon *et al*., 2005). With appreciation for the potential public health burden associated with mortality associated with rabies-related virus infection, several vaccine studies have investigated pan-lyssavirus vaccine cross-protection (Jallet *et al.,* 1999; Weyer *et al.,* 2007).

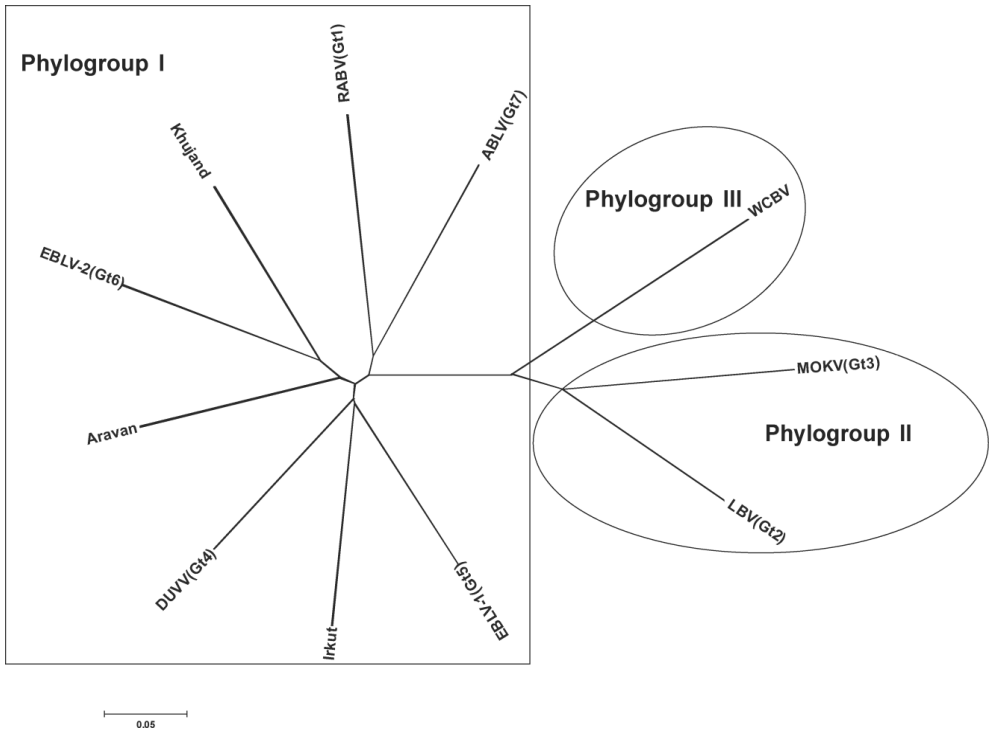

Figure 1.1: Phylogenetic tree of the *Lyssavirus* genus showing different phylogroups, reprinted with permission from Nel and Markotter, 2007.

1.3 Global epidemiology of rabies

Lyssaviruses have a distinct geographic spread. Rabies virus (RABV) occurs in all major continents except the Arctic regions and Australia. Lagos bat virus (LBV), Mokola virus (MOKV) and Duvenhage virus (DUVV) are found exclusively in Africa. European bat lyssavirus virus-1 and 2 (EBLV) occur in Europe. Australian bat lyssavirus (ABLV) is reported from Australia but seropositivity to ABLV in *Miniopterus schreibersi* has also been reported in the Philippines (Arguin *et al*., 2002). Rabies currently kills an estimated 55 000 people worldwide annually, with most of these deaths occurring in Africa and Asia (WHO, 2005). Most reported rabies cases can be attributed to RABV (formerly gt1), however rabies-related lyssaviruses have also been implicated in human deaths. MOKV infection was implicated in two human deaths in Nigeria, in 1969, although these reports and the diagnosis were not definitive (Familusi and Moore, 1972). In 1971 the first DUVV human fatality was reported in a man (a Mr Duvenhage) in the North West Province of South Africa who succumbed to a rabies-like illness after being bitten on the lip by a bat (Meredith *et al*., I97I). In 2006 another human succumbed to a DUVV infection in South Africa 30km away from the original human case (Paweska *et al.,* 2006), a third DUVV fatality was reported in a 34 year old Dutch tourist scratched by a bat while on holiday in Kenya (Van Thiel *et al.,* 2008). EBLV have been implicated in four recorded human cases (Fooks *et al.,* 2003). ABLV has also been reported to cause human deaths, in 1996 a 39 year old female died after being presumably bitten by a yellow-bellied bat (*Saccolaimus flaviventris*) (Allworth *et al*., 1996), a second ABLV case was reported in 1998 in a woman identified with ABLV encephalitis (Hanna *et al*., 2000). To date LBV, Irkut, Aravan, Khujand, WCBV and SHIBV) are the only lyssaviruses that have not yet been associated with human infection. The lack of epidemiological data on the African lyssaviruses and the newly isolated Eurasian bat lyssaviruses can be attributed to the lack of active surveillance in these regions of the world (Nel *et al*., 2005; Markotter *et al*., 2006a, b). The fact that some of these African lyssaviruses have been isolated in bat colonies around areas of dense human populations is a possible public health concern (Markotter *et al.,* 2006; Kuzmin *et al*., 2008).

1.4 The lyssavirus genome

The lyssaviruses have single stranded negative sense ribonucleic acid (RNA) genomes of approximate 11.9 kilo base pairs (kbs). These viruses have the characteristic bullet shape

of *Rhabdoviridae* family. The virion encodes five viral proteins in a 3'-5' direction: nucleoprotein (N); phosphoprotein (P); matrix (M); glycoprotein (G) and polymerases (L). All five proteins are present in virions (Poch *et al*., 1988). Non-coding intergenic regions are also present in the genome (Tordo *et al.,* 1986). The intergenic regions are usually a small number of nucleotides with the exception of the G-L intergenic region.

1.4.1 Lyssavirus antigens with vaccine application

This section discusses characteristics which relate to RABV but are also generally applicable to all rabies-related lyssaviruses.

1.4.1.1 The glycoprotein

The glycoprotein gene is transcribed into a 18s monocistronic messenger RNA (mRNA). The mRNA is translated into a 524 amino acid glycoprotein precursor comprising of a 19 amino acid (aa) signal peptide domain, a 439 aa ectodomain, a 22 aa hydrophobic transmembrane domain and a 44 aa cytoplasmic domain (Wiktor *et al.,* 1973; Tordo and Poch, 1988; Wunner, 2002). The signal peptide provides the signal for insertion of the glycoprotein into the membrane of the rough endoplasmic reticulum-golgi plasma membrane pathway. The mRNA glycosylation takes place at endoplasmic reticulum and thereafter the glycoprotein proceeds to the golgi-apparatus where it is covalently modified by inclusion of fatty acid chains. Upon insertion into golgi apparatus membranes, the signal peptide is cleaved off yielding the mature 505 aa glycoprotein that contains three asparagine-X-serine/threonine potential N-glycosylation sites in the extracellular domain (Anilionis *et al.*, 1981). Appropriate glycosylation of the glycoprotein is important for proper expression and function. Non-glycosylated glycoprotein is not expressed at the cell surface (Burger *et al.*, 1991; Shakin-Eshleman *et al.*, 1992) and is not effective as a vaccine (Yelverton *et al.*, 1983; Lathe *et al.*, 1984). Non-glycosylated glycoprotein lacks characteristic peplomers (spikes) and limits infection to initially infected neurons without spread of virus into secondary neurons (Etessami *et al.*, 2000).

Rabies virus glycoprotein protein is a type I transmembrane protein and forms characteristic spike-like peplomers on the virion. The peplomers comprise three homomers (3 x 65 Kilo Dalton (kDa) of glycoproteins (Whitt *et al.,* 1991; Gaudin *et al*.,

1992). The mature RABV glycoprotein comprises three distinct domains: an ectodomain (antigenic domain) which forms the head of the peplomers, a transmembrane domain that anchors the glycoprotein to the virion and a cytoplasmic domain that interacts with the matrix protein.

The glycoprotein has been shown to be the most potent inducer of viral neutralizing antibodies (Crick *et al*., 1969) and contains at least eight antigenic sites, I-IV, a, b, c and G1. Sites II and III are the major epitopes (Lafon *et al*., 1983). Site II is discontinuous spanning amino acid 34-42 and 198-200 with amino acid 35 and 198 conserved (Prehaud *et al.,* 1988). The two domains of site II are linked together by disulfide bridges between cysteine residues at amino acid 35 and 207. Site III is continuous spanning amino acid 333-338 (Seif *et al*., 1985). It has been shown approximately 97 % of monoclonal antibodies (mAbs) raised against the glycoprotein will bind to immunodominant sites II and III (Coulon *et al*., 1993), consequently the glycoprotein is used in subunit vaccines. Its been established that lyssaviruses glycoprotein domains can be exchanged without affecting infectivity of corresponding chimeric virus (Mebatsion *et al.,* 1995). Subsequent work by (Bahloul *et al*., 1998) showed that combining the glycoprotein antigenic sites II and III of different lyssaviruses raised VNAb against different lyssaviruses.

The glycoprotein is involved in host receptor binding (Kawai and Morimoto, 1994; Thoulouze *et al.,* 1998). Various receptors are used for viral entry into host cells such as nicotinic acetylcholine receptor at neuromuscular junctions that requires a peptide fragment between aa 189 and aa 214 on the glycoprotein, this receptor has a close association with a site II lysine residue 198 thus helps in viral invasion of host nervous system (Preheud *et al*., 1988; Lentz *et al*., 1982). The neuronal cell membrane p75 neurotropin receptor (p75NTR) is another receptor and interacts with phenylalanine 318 and histidine 352 on the glycoprotein to facilitate binding of RABV to (p75NTR) receptor (Langerin *et al*., 2002; Tuffereau *et al*., 1989). The low affinity nerve-growth factor receptor is also a major receptor used by the glycoprotein (Lewis *et al*., 2000).

1.4.1.2 The nucleoprotein

The nucleoprotein is 450 aa in length and is the most conserved antigenic protein between

different RABV strains and lyssaviruses based on amino acid sequence similarity (Dietzschold *et al.,* 1988; Kissi *et al.,* 1995). The nucleoprotein's main function is encapsulation of viral RNA and viral replication intermediates, hence protecting the genome from degradation by cellular ribonucleases (Tordo and Poch, 1988; Wunner, 2002).

The nucleoprotein protein contains genotype specific epitopes enabling distinction between different genotypes on basis of the reactivity pattern with anti-nucleoprotein monoclonal antibody panels (Schneider *et al.,* 1973; Smith, 1989). Moreover the nucleoprotein has some specific antigens that are present in all known lyssaviruses and that these antigenic epitopes are recognized by B and T helper cells (Goto *et al.,* 1995). Based on this rationale it was considered that inclusion of the nucleoprotein antigen in subunit vaccines will broaden the cross protective spectrum between lyssaviruses (Dietzschold *et al.,* 1987). Contrary to this anticipation, a MOKV nucleoprotein-glycoprotein chimeric DNA vaccine failed to improve cross protection between different lyssaviruses (Nel *et al.,* 2003). Independently the nucleoprotein confers protection against peripheral RABV challenge if administered pre-exposure but not post exposure (Fujii *et al.,* 1994). The mechanisms for pre-exposure protection can be largely ascribed to an anti-nucleoprotein antibody, this in turn induce an antigen-induced proliferative response of human and murine T cell lines (Celis *et al.,* 1985; Takita-Sonada *et al.,* 1993). Anti-nucleoprotein antibodies might act directly on the virus replication since anti-nucleoprotein antibodies have been reported to inhibit virus replication *in vitro* (Lafon and Lafage, 1987). The role of the cytokine, interferon (IFN) in complementing protection afforded by the nucleoprotein is also important as demonstrated in a comparative study with mice depleted of IFN and immunized with the nucleoprotein (Dietzschold *et al.,* 1989).

Rabies virus nucleoprotein has been implicated as a superantigen that evades host immune response against RABV by binding (without processing) simultaneously to major histocompatibility complex class II (MHC II) and variable region of T-cell receptor (TCR) b-chain (Lafon *et al*., 1992; Lafon, 1993). This interaction subsequently stimulates T-cells to release cytokines causing systemic shock (Fraser *et al.,* 2000). Interestingly recent studies with bacterial superantigens selectively mutated at TCR-binding site have suggested a

novel vaccine application of superantigens. These superantigens can be employed as carrier proteins targeting antigen presenting cells by binding MHC II, thus enhancing antigenicity and immunogenicity of conjugated MHC I peptide antigen (Loh, 2006).

1.5. Immune responses against rabies virus

RABV has adopted a stealth strategy to survive and complete its replication. The virus avoids the host immune response by maintaining a strict neurotropic nature with no viremia. Infected nerve tissue avoids immune surveillance by stimulating molecules that destroy T cell activity using calcitonin gene related peptides. Several molecules are used to destroy migrating T cells; such include FasL, HLA-G and B7-H1 (Bahloul *et al*., 2004; Lafon *et al.,* 2007). FasL is a 40kDa type II surface glycoprotein ligand that binds to Fas/CD95 expressed on T cells. This association induces a caspase-dependent apoptosis of T-cell (Lafon *et al.,* 2005). The HLA-G mediates destruction of T cell and natural killer (NK) cells by ligating cell receptors (Lafon *et al*., 2004). RABV infection in CNS results in up-regulation of B7-H1, this is a ligand that binds to the inhibitory molecule PD-1. The association provides an inhibitory function to T and B cells (Lafon *et al*., 2010). B7-H1 deficient mice have been shown to be more resistant to acute rabies infection. Due to RABV immuno subversion, post-exposure prophylaxis should be administered immediately after a bite since it uses the short period of time before the onset of immuno evasion so as to activate T cell in the central nervous system (CNS) (Roy and Hooper, 2007). Rabies virus triggers strong immune response in periphery (Vuaillat *et al.,* 1994; Roy *et al.,* 2007), because of this the immunization is usually done intramuscularly (i.m) or intradermally.

The main components that confer an immune response to the RABV antigens are viral neutralizing antibodies (VNAb) (antibody mediated immunity) and cytolytic T lymphocytes (CTL) (cell mediated immunity) (Perry and Lodmell, 1991). The VNAb are involved in clearance of virus before the virus can spread to the CNS, in neutralizing virus released from infected cells lysed by cytolytic responses, binding onto virions and mediating virolysis (Shanker *et al.,* 1991). The VNAb also stimulates interleukin-4 (IL-4) production which complements antibody mediated immunity (Solomon *et al*., 2002).

The CTL acting as T helper (Th) cells (CD4) can induce antibodies against a viral infection,

alternatively as cytotoxic T cells (CD8) help in lysis of infected cells (Lafon *et al.,* 2003). T cells have been shown to be crucial in controlling RABV neuroinvasiveness (Galelli *et al.,* 2000; Xiang *et al.,* 1995; Hooper *et al.,* 1998). CD4 and CD8 (with CD8+ dominating) are involved in apoptosis of infected cells. The CD 8 T cell recognizes virus loaded on MHC I (Lafon, 2005). T cells can also clear infection from CNS by secreting antiviral cytokines such as interferon γ (INFγ) (Kondig *et al*., 1993; Chesler *et al*., 2002). INFγ induces synthesis of type I nitrogen oxide synthase that suppresses viral transcription (Komatsu *et al.,* 1996).

The glycoprotein is the most important antigen for induction of antibodies with sites II and III as the most potent (Lodmell *et al.,* 1998). These immunodominant sites are recognized by both VNAb and CTLs (Dietzschold *et al*., 1990). The nucleoprotein is also involved in immunogenicity (reviewed in Fu *et al.,* 1991a). The N antigen has both T helper and B cell epitopes that are associated with CTL responses (Dietzschold *et al*., 1987; Ertl *et al*., 1989).

Several regions distributed along the glycoprotein ectodomain are involved in the induction of T helper cells (MacFarlan *et al*., 1984; Wunner *et al*., 1985). Usually antibodies/immunoglobulin (IgG) present a "bridge" linking antigens to Fc receptors (FcRs) on phagocytes and other immune cells (reviewed in Woof *et al.,* 2004) and regulate antibody-mediated responses (reviewed in Nimmerjahn *et al*., 2008). Apart from NK cells which express only a low affinity for activating FcR, other innate immune effector cells co-express both activating and inhibitory FcRs. The differential binding affinities of the antibody isotypes IgG1/IgG2a, and subsequent signaling cascade, determines the magnitude of the induced antibody response. With respect to mice, IgG1 antibodies bind to the inhibitory FcR, Fc_RIIB, with a much higher affinity than to activating FcR, which, for IgG1, is Fc_RIII (Nimmerjahn *et al*., 2005). Due to a higher binding efficiency to Fc_RIIB than to Fc_RIII, IgG1 provides a dampening effect which limits the resulting effector functions. Conversely, IgG2a binds with much higher affinity to activating Fc_RIV than inhibitory Fc_RIIB. In addition, it appears that Th1 induction increases the expression of both IgG2a and activates Fc_Rs (Fc_RI and Fc_RIV), thus amplifying the IgG2a-mediated effector function. This is consistent with the findings that IgG2a antibodies are potent inducers of anti-viral effector functions. Hence a vaccine inducing potent IgG2a antibodies, while minimizing IgG1 responses, might prove beneficial.

1.6 Vaccinia virus as vaccine carrier

Vaccinia virus belongs to a large group of double stranded deoxyribonucleic acid (dsDNA) poxviruses in the family *Poxvirida*e and subfamily *Chordopoxvirinae* (Fauquet *et al*., 2005). This subfamily is subdivided into eight genera, *Avipoxvirus*, *Capripoxvirus*, *Leporipoxvirus*, *Molluscipoxvirus*, *Orthopoxvirus, Parapoxvirus*, *Suipoxvirus* and *Yatapoxvirus*. The genus Orthopoxvirus has been extensively studied with vaccinia virus as the prototype virus in this genus. Poxviruses have been successfully applied in history to eradicate smallpox. On may 14 1776 Edward Jenner demonstrated that by inoculating 8 year-old James Phipps with cowpox pustules, the boy was resistant to smallpox and the procedure became known as vaccination, cowpox virus was later superseded by the vaccinia virus (Jenner, 1798).

The genome of vaccinia virus Copenhagen strain (Goebel *et al*., 1990) (accession number M35027), modified virus Ankara (MVA) (Antoine *et al*., 1998) (accession number U94848), Tian Tan (accession number AF095689) and Western Reserve (WR) (accession number AY243312) (Smith *et al*., 1991) have been sequenced. These sequences reveal a highly conserved central region of 100 kb encoding genes necessary for viral replication (Gubser *et al*., 2004). The terminal genes are largely non-essential for viral replication in cell culture and encode proteins involved in host range and virulence. Gene expression is regulated in a temporal cascade of three gene classes in which expression of one gene class is dependent on prior expression of genes in the previous gene class (reviewed in Broyles, 2003). The early class of genes represents almost half of viral genome and is transcribed by virus DNA-dependent RNA polymerase. Early genes encode proteins involved in DNA replication like DNA polymerase as well as immunomodulators that block innate antiviral defenses, this property is exploited in vaccine development. Once DNA replication begins, intermediate gene expression starts, these encode proteins required in late gene expression such as late transcriptions factors. The late class of genes encodes proteins needed in making new virions and enzymes packaged within virions for next round of infection. Two infectious forms of virus which are structurally and antigenically different are present namely, intracellular mature virus (IMV) and extracellular enveloped virus (EEV) (Boulter and Appleyard, 1973). The IMV represent majority of infectious viral progeny while EEV mediate long range spread of virus in cell culture. Some IMV particles leave viral factories and are transported on microtubules to the microtubule organizing

center (MTOC) (Sanderson *et al.,* 2000). In MTOC IMV become enwrapped in a double membrane derived from *trans*-golgi network and the resultant virus is referred to as intra cellular enveloped virus (IEV) (Schmelz *et al*., 1994). IEV get transported to cell periphery where they fuse cell membrane to release the virions as EEV.

Several properties of poxviruses generally render them potential candidates as vaccine vectors. A clear benefit of vaccinia virus is the ability of the virus to infect a wide host range - vaccinia virus infects most cell lines of mammalian origin (variable efficiency). Poxviruses have a distinctively large genome of approximately 200 kb that can accommodate foreign DNA inserts up to 25 000 base pairs (bp) while still remaining stable (Smith and Moss, 1983). Vaccinia virus also replicate autonomously without using host replication machinery. Poxviruses are the only DNA viruses known to replicate within cytoplasm (Bonnet *et al.,* 2000). Multiple independent antigens can also be simultaneously expressed in these systems (Perkus *et al.,* 1985). Because transcription of vaccinia virus genes is carried out by virus-encoded enzymes in the cytoplasm and splicing of RNA does not occur, there are requirements for vaccinia virus promoters and uninterrupted open reading frames. Vaccinia virus thus finds an application as a vaccine platform with the capacity to be engineered genetically to alter its pathogenicity and produce recombinant subunit vaccines. Recombinant vaccine gene expression by vaccinia virus was first done in 1982 and has since become a standard protocol in most laboratories (Mackett *et al.,* 1984).

1.6.1 Host restricted and attenuated vaccinia viruses as recombinant vaccine vectors

The use of replication competent vaccinia virus based-vaccines has raised safety concerns with a potential to induce disease. This has severe implications in immunocompromised individuals (Fenner *et al.,* 1988; Fenner *et al.,* 1989). The health concern associated with vaccinia virus have led to a continued search for better alternatives with reduced virulence while still retaining the capacity to replicate and high gene expression-the so called “new generation poxvirus vectors” (Moss *et al.*, 1996). Table 1.1lists some of these vectors

Table 1.1 Comparisons of some poxvirus recombinant vaccine vectors

Vector	Description	References
Modified vaccinia virus Ankara (MVA)	Third generation vaccinia virus vaccine passaged 570 times in primary chick embryo fibroblasts to produce a mammalian cell replication defective virus with genomic deletion equivalent to 31 000 bp	Meyer *et al.*, 1991
The Lister clone 16m8	Third generation vaccinia virus vaccine, was developed in Japan in 1975 by passaging the Lister strain through primary rabbit kidney epithelial cells at low temperature (30 °C)	Kenner *et al.*, 2006
The Dairen 1 strain	Third generation vaccinia virus vaccine obtained by 13 serial passages of parental strain in 1 day old eggs	Tagaya *et al.*, 1961
NYVAC	Fourth generation vaccinia virus vaccine derived from vaccinia virus Copenhagen strain and contains a deletion on 18 open reading frames (ORF) involved in host virulence	Tartaglia *et al.,* 1992; Paoletti *et al.,* 1996
lumpy skin disease virus Neethling strain LSDV-SA	passaged 61 times on lamb kidney monolayer then 20 times in chorioallantoic membranes of chicken embryos plus a final 3 passages on lamb kidney cells	Van Rooyen *et al.*, 1969
ALVAC (canarypox)	Kanapox (Avipoxvirus) attenuated by serial passage in embryonated eggs	De Bruyn *et al.,* 2004; Weli *et al.*, 2005

Recent studies using recombinant vaccinia virus expressing full-length RABV, MOKV and WCBV lyssavirus glycoprotein failed to confer cross protection against heterologous challenges. The study also concluded that both single and double antigen combinations had comparable VNAbs (Weyer, 2006). In the same study efficacy of MVA expressing full-length RABV glycoprotein was evaluated, MVA could only be used in prime boost regimen administered i.m and lacked substantial humoral response when administered orally. The

insufficient humoral response can be ascribed to the lack of circulating antigen (no infectious progeny) due to severe attenuation of these recombinant viruses. Conversely, the MokG vaccine (full length MOKV glycoprotein) provided cross-protection against a LBV challenge (Weyer *et al*., 2007). These findings were in agreement with preceding studies by Bahloul *et al.* (1998) who used chimeric glycoprotein constructs of MOKV and RABV PV strain, respectively MokPV and PVPV. The authors made use of a highly efficient eukaryotic expression vector pCI-neo in which chimeric constructs of MOKV site II and RABV Pasteur strain (PV) site III were cloned. These biologics protected against gts 1 and 3 lyssaviruses, cross protected against gts 2 and 6 lyssaviruses and only partially cross protected against gt4 and gt5 lyssaviruses (Jallet *et al.,* 1999).

1.6.2 Immune responses against vaccinia virus

Vaccinia virus has been shown to induce potent immune responses modulated by both innate and humoral immune responses. In murine models vaccinia virus induces massive $CD8^+$ T cell activation with almost 30-80% spleenic $CD8^+$ T cells being virus specific (Butz and Bevan, 1998). The vaccinia virus specific $CD4^+$ and $CD8^+$ effector T cells were detected at 2 weeks after primary vaccination (Amara *et al*., 2004). Upon reaching the peak of response a long lived pool of memory $CD8^+$ T cells develops (Demkowicz *et al*., 1996). Its also been shown that the virus specific $CD8^+$ pool contracts after viral clearance by host immune responses and remaining cells differentiate into long term memory T cells (Murali-Krishna *et al*., 1998). However long term maintenance of effector T cells has been shown to be antigen dependent (Crotty *et al*., 2003; Demkowicz *et al*., 1996) thus citing a supplementary advantage to using a replication competent vector such as vaccinia virus Copenhagen strain. At the peak of response the effector CD8+ T cells display an activated phenotype, proliferate and produce INF γ in response to antigen (Butz and Bevan, 1998). Consistent with effector function $CD8^+$ T cells express perforins and granzymes B which are hallmarks of effector T cells with a high cytolytic activity (Rock *et al*., 2005). Studies have demonstrated that some of the memory cell markers on activated $CD8^+$ T cells can be reactivated providing a basis for the long term immunity (Miller *et al.,* 2008).

1.7 Expression systems for rabies virus glycoprotein

Eukaryotic and prokaryotic expression systems have been used to express RABV

glycoprotein (Dietzschold *et al.,* 1996). During expression of foreign antigen in a production host, suitable promoters increase production levels (Suarez *et al*., 1997). A major problem has been proteolytic degradation of foreign protein. Prokaryotic expression systems have a setback as are unable to carry out post translational modification (Reviewed in Sissela *et al*., 1999). Several strategies to avoid this problem are being investigated, given the advantages an effective prokaryotic system may provide.

Whole virus recombinant subunit vaccines have been a relatively new development in vaccinology (Andersson *et al.,* 1998). Replication deficient human adenovirus 5 with deletions at E1 into which the RABV glycoprotein was inserted was able to elicit immune response in mice (Xiang *et al.,* 1996). Although this system induced antibody response greater than equivalent vaccinia virus as shown by (Xiang and Ertl, 1999), use of replication-deficient adenovirus has been hampered by the fact that pre-existing antibodies to human adenovirus can interfere with vaccine efficacy (Eric *et al.,* 1999). Excretion of vaccine virus into the environment is another setback arguing against use of adenovirus vectors (Tatsis and Ertl, 2004). The baculovirus expression system Autographa califonia nuclear polyhedrosis virus has also been used to express RABV and MOKV glycoproteins in *Spodoptera frugiperda* (Sf9) cell culture. The resultant glycoprotein was immunogenic and conferred protection in mice (Prehaud *et al*., 1989). The poxvirus system has been extensively employed to express RABV glycoprotein. The live replicating raccoonpoxvirus (Cadoz *et al*., 1992), recombinant canarypoxvirus (Taylor *et al*., 1990) and vaccinia MVA strain (Weyer *et al*., 2007) are among the many poxviruses used.

Although replication competent vaccines theoretically pose a potential safety concern, such vaccines still add value to vaccine repertoires. The vaccinia virus Copenhagen strain is a potential candidate with this respect. Attenuated by insertion of foreign gene into TK region, virulence of even the most neurovirulent strains is greatly reduced (Buller *et al*., 1985). Nevertheless, this attenuation does not compromise viral replication at site of inoculation. Thus vaccinia virus Copenhagen strain mimics a natural infection by inducing viral neutralizing antibodies for prolonged period of time.

2. Objectives of the study

This study evaluated the cross protective capacity of recombinant vaccinia virus Copenhagen strain expressing either chimeric or full length lyssavirus glycoprotein genes. Two chimeric glycoprotein genes previously constructed by Jallet *et al*., 1999, were evaluated. Firstly a PVPV chimeric construct carrying both glycoprotein antigenic sites II and III of RABV and secondly a MokPV chimeric construct carrying glycoprotein antigenic sites II of MOKV and III of RABV. A full length glycoprotein gene (MokG) was also included in this study, the construct was previously constructed in (Weyer, 2206). This construct contained an uninterrupted glycoprotein gene of MOKV.

The transfer vector pGVWR1-gpt containing MokPV, PVPV or MokG glycoprotein genes was homogonously recombined within the vaccinia virus Copenhagen TK region. The TK region was chosen for insertion of foreign genes because it codes for an amino acid that is non-essential in replication in cell cultures and has been commonly used to insert foreign genes (Dubbs *et al*., 1964). Vaccinia virus Copenhagen strain has a supplementary benefit as an effective carrier of the RABV glycoprotein and is comparable to V-RG vaccine which has been successful as an oral vaccine in wildlife.

- To isolate homogenous recombinant vaccinia virus Copenhagen strain expressing chimeric glycoprotein genes (MokPV and PVPV) and full length glycoprotein genes (MokG)

- Evaluate VNAbs induced by a replication competent vaccinia virus Copenhagen strain expressing chimeric lyssavirus glycoprotein genes (MokPV and PVPV) and full length glycoprotein gene (MokG). VNAbs induced by recombinant vaccine viruses were tested against RABV, MOKV and LBV.

Chapter 2

The construction and isolation of recombinant vaccinia virus Copenhagen strain expressing various lyssavirus glycoprotein genes

2.1 Introduction

Vaccinia virus has in recent years become a popular tool in molecular biology particularly due to its benefits as mentioned in section 1.5. However the use of live vaccinia virus vaccines has been a sensitive issue in vaccinology due to possibility of vaccine virus reverting to virulence in the case of attenuated vaccines or residual live virus in killed preparations (Perkus *et al*., 1995b). Vaccinia virus also has been reported to induce disease in immunocompromised vaccinees and possible spread to unexposed individuals (Jacobs *et al*., 2009). To circumvent such limitations, highly attenuated strains of vaccinia virus have been developed as safe vaccine platforms. Such replication deficient poxviruses include the orthopoxviruses: MVA and NYVAC, and avipoxviruses: fowlpox and canarypox (ALVAC). Prime-boost regimens including different combinations of these non-replicating strains have become routine to enhance immunity and to avoid complications with preexisting immunity to vaccinia virus vectors (Webster *et al*., 2005; Woodland, 2004). These vectors induce some level of protection against many heterologous agents when used as a vaccine in both human and veterinary medicine (Bisht *et al*., 2004; Bublot *et al*., 2007; Chen *et al.,* 2005; Kreijtz *et al*., 2007; Minke *et al*., 2007; Poulet *et al*., 2007; Ramos *et al*., 2008). Despite being capable of inducing protective immunity against a variety of pathogens, their limited replication still generates concern as to their ability to induce an immune response as effective as their replication-competent counterparts (Abaitua *et al*., 2006; Dai *et al*., 2008; Karkhanis and Ross, 2007). Studies comparing replication-competent and non-replicating vaccine vectors clearly demonstrate that in attempting to improve safety, long term protection and efficacy are often lost. Vaccinia virus Copenhagen strain has been successfully used in oral vaccine campaigns to eliminate rabies in wildlife (Pastoret and Brochier, 1993) and was subsequently selected as the vaccine vector of choice in the current study.

The construction of the chimeric glycoprotein genes (MokPV and PVPV) has been described previously in (Bahloul *et al.,* 1998). In that study authors cloned the chimeric glycoprotein genes (MokPV and PVPV) into an expression vector. The chimeric MokPV contained a glycoprotein gene reconstituted from two partial glycoprotein genes, the first half (amino acids -19-257) was derived from MOKV Ethiopian isolate (accession number U17064) and the second half (amino acids 258-503) was derived from RABV Pasteur strain. The chimeric PVPV construct contained a glycoprotein reconstituted from two partial glycoprotein genes of the RABV Pasteur strain. The first half consisted of amino acids -19-252 and the second half amino acids 253-505. In both chimeras, the first half represented the N-terminal of the glycoprotein gene and contained glycoprotein antigenic site II whilst the second half represented the C-terminal which contains glycoprotein antigenic site III. In The amino acid region 253-275 overlaps the only non-conformational epitope (VI) and is presumably less structurally constrained than sites II and III. This region was therefore chosen as a "flexible hinge" between the two sites of the chimeras.

To be able to use the same chimeric glycoprotein constructs in a vaccinia Copenhagen vector, a transfer vector pGVWRI-gpt containing sequences homologous to the vaccinia virus TK gene was employed. Briefly, this pGVWRI-gpt transfer vector contains a vaccinia p7.5 late promoter, E.coli guanyl phosphoribosyl transferase (gpt) gene, an enhanced green fluorescent protein (EGFP) gene and the multiple cloning site (MCS) all flanked by sequences homologous to the TK gene of vaccinia virus. The chimeric constructs (MokPV and PVPV) were digested out of the pCIneo plasmid and ligated into the MCS of the vaccinia transfer vector (Weyer, 2006). In the same study the construction of the full length MokG construct was described. Briefly, the full length MOKV glycoprotein gene was digested from the recombinant construct, pBudCE4-MokG and ligated into the MCS of the transfer vector. The details of the pGVWRI-gpt plasmid are given in appendix D (adopted from Weyer, 2006).

Previous studies on DNA vaccine cross protection between phylogroups I and II showed that the chimeric MokPV construct was capable of cross protecting between phylogroups I and II (Jallet *et al*., 1999). The MOKV site III in the MokPV chimeric construct induced cross protection against LBV and protected against MOKV. The RABV site III in the same

construct induced partial cross protection against DUVV and EBLV-1 while fully protecting against RABV. Based on these findings, the MOKV site II and RABV site III were used in this study in anticipation that similar pan-lyssavirus cross reactive patterns will be achieved. Moreover the choice of vector –vaccinia virus Copenhagen strain was expected to induce long lasting VNAbs. On that note, site III of RABV has a supplementary benefit as a potent inducer of Th1 cells. The RABV site III induces VNAbs predominantly of the IgG2a subtype. This subtype has been shown to be more effective against RABV than IgG1 (Cenna *et al*., 2008). Secondly RABV site III was chosen as it has been demonstrated to be important in the presentation of heterologous site II of other lyssaviruses in a conformation recognizable by VNAbs.

Generally, selection of recombinant viruses is mediated by expression of a marker gene co-integrated into viral genome (Chakrabarti *et al*., 1985). The *Escherichia coli* (*E.coli)* enzyme guanyl phosphoribosyl transferase (gpt) was used in this study. The gpt gene (in the presence of xanthine and hypoxanthine) provides resistance against mycophenolic acid (MPA), a mycotoxin that inhibits purine metabolism (Boyle and Coupar, 1988). MPA inhibits the enzyme inosine monophosphate dehydrogenase and thereby prevents the formation of xanthine monophosphate. This results in the intracellular depletion of purine nucleotides and in an inhibition of cell growth (Mulligan *et al.,* 1981). The recombinant virus was grown in selection media containing MPA and only recombinants containing *E. coli* gpt gene grow in selection media supplemented with xanthine and hypoxanthine (Falkner and Moss, 1988).

It should be highlighted that this technique (homologous recombination) is particularly difficult to carry out, and although recombination occurs at a relatively high rate in poxvirus infected cells, almost the entire progeny virus remains parental wild type virus (99%) (Moss, 1992; Smith and Mackett, 1992). Once recombinants had been generated, they were purified from wild type viruses. Several techniques for the isolation of homogenous recombinant vaccinia viruses have been described (Isaacs, 2004). In this study the transient dominant selection of recombinant vaccinia viruses was used with some modification to the protocol. This was accomplished by applying selective pressure determined by markers (*E.coli* gpt gene) encoded on the recombinant virus *in vitro*. An

improvement to the technique involved incorporation of the enhanced green fluorescent protein gene into DNA transfer vector (pGVWRI-gpt) to help with the selection process. The plaque selection was a tedious process and contamination with wild type viruses frequently happened. To minimize contamination, foci were picked from highest dilution well with a single focus. To ensure complete removal of parental virus, several consecutive rounds of plaque purification were done (at least five). Once plaques were confirmed to be homogenous by PCR, selection pressure was lifted. The possibility of recombinant viruses reverting back to wild type once selection pressure had been lifted was another challenge.

This chapter describes the generation, purification and isolation of homogenous recombinant vaccinia virus Copenhagen strain expressing chimeric glycoprotein genes of MOKV and RABV.

2.2 Materials and methods

2.2.1 Viruses, plasmids and genes

The vaccinia virus Copenhagen strain used in this study was obtained from Dr. C.E. Rupprecht (Centers for Disease Control and Prevention (CDC), United States of America). The pCIneo constructs containing chimeric lyssavirus glycoproteins genes of RABV and MOKV was obtained from Dr Tordo (Laboratoire des Lyssavirus, Institute Pasteur, Paris, France) (Jallet *et al*., 1999). The details of vaccinia virus transfer vector, pGVWR1-gpt are described in (Weyer, 2006). The human rabies immunoglobulin (HRIG) used in RABV and MOKV glycoprotein gene expression analysis, was provided by Dr. Jacqueline Weyer (NICD-NHLS), South Africa.

2.2.2. Cloning chimeric lyssavirus glycoprotein gene in vaccinia virus transfer vector pGVWRI-gpt and transformation of competent cells

The details of the molecular cloning of chimeric lyssavirus glycoprotein genes (MoKpv and PVPV) in the pCIneo plasmid are described in a previous study (Jallet *et al*., 1999). The chimeric glycoprotein coding sequences were digested from the pCIneo contruct and ligated into the vaccinia virus transfer vector -pGVWR1-gpt to generate MoKPV-pGVWR and PVPV-pGVWR respectively for MoKpv and PVPV chimeric constructs (Weyer, 2006). In

the same study a full length MOKV glycoprotein gene was digested from the recombinant construct, pBudCE4-MokG and ligated into the MCS of pGVWR1-gpt vector to generate MokG-pGVWRI plasmid. The enhanced green fluorescence protein (EGFP) gene was also cloned into MokG-pGVWRI and MoKPV-pGVWR (Weyer, 2006). The plasmids were then transformed in competent *E.coli* (JM109 strain) as described in (Weyer, 2006). The position of gene inserts was verified by molecular sequencing as described in 2.3.2.

2.2.3 Generation of recombinant vaccinia virus Copenhagen strain

Vero cell cultures (African green monkey cell line, American Type Culture Collection reference number, CCL-81) were transfected in six well cluster cell culture plates (Nalge Nunc International, USA). A rich cell culture medium containing Dulbecco's Modified Eagle's Medium and Ham's F12 medium prepared in a 1:1 ratio, hereafter DMEM/F12 was used (Lonza, USA). The media also contained 4 mM L-glutamine and 15mM hepes buffer. Plates were seeded with approximately 2 X 10^5 Vero cells maintained in DMEM/F12 containing 10 % fetal calf serum (FCS) and supplemented with 0.75 mg/ml antibiotics (penicillin, 100 units/ml; streptomycin, 100 µg/ml and amphotericin B, 0.25 µg/ml) (all from Lonza). Vero cells were then incubated 24hrs in a humid chamber at 37 °C and 5 % CO_2. Confluent monolayers were infected with vaccinia virus Copenhagen strain at a multiplicity of infection (m.o.i) of 1.0. Some wells were kept uninfected, to act as a background controls in the immunofluoresence assay. The virus was allowed to absorb for 90 minutes at 37 °C and 5 % CO2. During the incubation the transfection complexes were prepared with the recombinant plasmids MokG-pGVWRI, MoKPV-pGVWR or PVPV-pGVWR. Plasmid DNA was purified using spin columns (QIAquick® Gel extraction Kit, QIAGEN) and analyzed spectrophotometrically for purity and concentration. 1 µg of DNA with a A260/A280 ratio of 1.8 were used for the preparation of the transfection complexes with the transfection reagent, FuGene® (Roche Molecular Biochemicals) according to the manufacturer's suggestions. After virus absorption period the medium was aspirated from the cell monolayers and the cells washed with sterile phosphate buffered saline (PBS) to remove residual unabsorbed virus. The cells were fed with complete MEM with 2 % FBS. The transfection complexes were then added to the cell cultures and cell cultures incubated under standard growth conditions up to 3 hrs, with subsequent removal of inoculums

followed by washing of monolayers with PBS and replacement of complete medium. The plates were then incubated under standard growth conditions for another 48 hrs.

2.2.4 Purification of recombinant virus clones

Six well plates were seeded with 2 x 10^5 cells/ml in total media 2ml, (Lonza) supplemented with 10% FCS 2mM glutamine and 1% of penicillin (100 units/ml)/streptomycin (100 ug/ml) amphotericin B (0.25 ug/ml) (Lonza, USA). The cells were incubated 24 hrs at 37 °C and 5 % CO_2 until 80 % confluent. The media was aspirated and replaced with selection media (DMEM/F12 containing 2% FCS and supplemented with 25µg/ml mycophenolic acid, 250 µg/ml xanthine and 15 µg/ml hypoxanthine (all from Sigma Aldrich, Germany). The cells were incubated 8hrs in selection media. During incubation virus serial dilutions were prepared in selection media in range of 10^{-1} to 10^{-3} in total volume 1ml. When incubation was finished virus dilutions were pipetted into 6 well plate with one well left uninfected to serve as a negative control followed by a 5hr virus adsorption. Virus material was aspirated after 5 hrs and replaced with 2ml of selection media. Plates were incubated 48-72 hrs at 37 °C and 5 % CO_2 until visible cytopathic effects (CPE) upon which 40 µl of virus plaques are picked by pipetting into microcentrifuge tubes containing 160 µl selection media. The contents were analyzed by PCR to determine homogeneity of recombinant virus. Once recombinant virus was confirmed to be homogenous, remaining half volume of same batch was used to infect Vero cell monolayer in a 6 well plates as described before (section 2.2.3) and virus passaged in selection (DMEM/F12 containing 2% FCS and 25µg/ml mycophenolic acid supplemented with 250µg/ml xanthine and 15µg/ml hypoxanthine) at least three more times before lifting selection and growing virus in growth media without selection (DMEM/F12 containing 10 % FCS and 1% streptomycin and amphotericin).

2.2.5 Determining homogeneity of passage virus

2.2.5.1 Extraction of viral DNA

Extraction of genomic DNA followed the method of (Esposito *et al.,* 1981). Recombinant virus was harvested by freeze thawing three times followed by a brief centrifugation at 12 100 *g* on a benchtop centrifuge. Half volume of harvested sample were incubated 2 hrs at

56 °C with an equal volume of poxvirus lysis buffer (1% sodium dodecylsulphate, 20mM β-mercaptoethanol, 20mM ethylenediaminetetraacetic acid) and 100µg/ml proteinase K (Sigma Aldrich, Germany). Phenol and chloroform (Merck, South Africa) was used to separate viral DNA from degraded proteins according to (Sambrook and Russell, 2001). Equal volume phenol/chloroform/iso-propanol (25:24:1) was added with gentle mixing. Samples were then centrifuged 5 minutes at 12 100 *g* at 4 °C followed by careful transferring of the top aqueous layer into a microcentrifuge tube. Half the volume of chloroform:isopropanol (24:1) was added and contents again centrifuged 5 minutes at 14 800 g at 4 °C. Again the top aqueous layer was transferred into a microcentrifuge tube. DNA was precipitated in two volumes ice cold 100 % ethanol (Merck, South Africa) containing one-tenth of the volume 2.5M NaOAc (PH 5.3), DNA was precipitated 24 hrs at -20 ^{o} C) and pelleted by centrifugation at 12 100 *g* for 25 minutes. The supernatant was discarded and pellet air dried and resuspended in 10-20 µl nuclease free H_2O (Promega, USA) and stored at -20 ^{o} C.

2.2.5.2 PCR amplification of recombinant virus DNA

The Gene Amp PCR system 2700 (Applied Biosystems) was used in DNA amplification. The resuspended pellet was used as template in PCR reaction using primers (Vac Tkl TCCCATCGAGTGCGGCTAC and Vac Tkr GTCCCATCGAGGTGCGGCTAC) annealing at sites flanking inside the vaccinia virus genome thymidine kinase gene (Weyer, 2006).

PCR master mix:

1µl of DNA extraction (80 ng/ µl) was used as template
Expand High Fidelity PCR buffer (10) with $MgCl_2$ (Roche, Germany)
Expand High Fidelity polymerases (2.625U) (Roche, Germany)
10mM dNTP,s (2.5 of each) (Promega, (USA)
10pmol of each primer (VacTK1 and Vac TKr) (Integrated DNA Technologies, RSA)
The volume was adjusted to 50µl with nuclease free H_2O.
The negative control contained all contents of PCR mix without template.

The PCR cycles consisted of a 1 minute denaturation at 94 ^{o}C followed by 35 cycles of 30 seconds at 94 ^{o}C, 40 seconds at 50 ^{o}C and 3 minutes at 68 ^{o}C and a final incubation of 7

minute at 68 °C. The reactions were kept at 4 °C until analyzed on agarose gel. PCR product was analyzed on 1.0 % agarose gel.

2.2.5.3 Purification of PCR product

Once PCR amplicons were confirmed to be of the desired size, the DNA was purified from the PCR mixture. The Wizard SV and PCR Clean-Up System (Promega, USA), was used according to the manufacturer's protocol. Ten µl of membrane binding solution was added per 10 mg of excised agarose gel containing 40 µl PCR product and the mixture incubated at 65 °C until the gel slice was completely melted. The gel slice mixture was added to a column assembly and the DNA was bound to the silica contained within this assembly by centrifuging the assembly at 13 000 *g* for one minute. The bound DNA was washed with membrane wash solution twice and then eluted into 50 µl nuclease-free water and stored at -20 °C until sequencing.

2.2.5.4. Sequencing of PCR amplicons

The integrity of homologous recombination sites within vaccinia virus Copenhagen vector as well as position of promoters within the expression cassette was verified by DNA sequencing using vac TKr and vac TKl primers (see sequence in section 2.3.2). The DNA concentration from the agarose gel was estimated using a NanoDrop 2000 (ThermoScientific, USA) spectrophotometer. The reaction contained:

2 µl 2.5X Terminator mix (BigDye Terminator 3.1, Applied Biosystems USA)

1 µl 5X sequencing buffer

3.2 pmol of either the Vac Tkl primer or the Vac Tkr primer

[x] Amount template (approximate size of PCR product/10 = amount of µg to add).

Nuclease free water to make a 10 µl reaction

Sequencing PCR cycles: 1 minute denaturation at 94 °C followed by 25 cycles of 10 seconds at 94 °C, 5 seconds at 50 °C and 4 minutes at 60 °C.

2.2.5.5 Purification of sequencing product

The reactions were done in total volumes of 10 µl. one micro litre of 125mM EDTA was pipette into a micro centrifuge tube followed by the sequential addition of 1 µl 3M sodium acetate and then 25 µl 100 % non-denatured ethanol. The contents were briefly vortexed and incubated 15 minutes at room temperature. Following incubation the contents were centrifuged at 12 1000 *g* for 25 minutes. The supernatant was carefully removed and discarded. Hundred micro litres of 70% ethanol were then pipette into the micro centrifuge followed by centrifugation at 12 1000 *g* for 13 minutes. Again the supernatant was removed and discarded, the pellet was air-dried overnight and submitted for sequencing at the DNA sequencing facility at the Faculty of Natural and Agricultural Sciences, University of Pretoria. The nucleotide sequences obtained were edited using BioEdit Sequence Alignment Editor (version 7.0, Hall, Isis Pharmaceuticals, Icn 1997-2004).

2.2.6 Expression analyses (Indirect immunofluoresence assay)

Confluent monolayers of Vero cells were grown on 8 chamber slides (Labtek Nalge Nunc USA). Semi purified virus material was thawed then briefly centrifuged to pellet cell debris. Hundred µl of virus material was used to infect Vero cells on 8 chamber slides. MOKV and RABV CVS strain were used as positive controls followed by 48-72 hr incubation at 37 °C and 5 % CO_2 in humidity. Some wells were left uninfected as negative controls. Upon completion of incubation culture medium was removed, the plastic wells removed from the glass slide and the cells fixed in ice cold acetone (Merck, South Africa) for 10 minutes. Slides were flooded with standard human rabies immunoglobulin (SRIG) as a primary antibody (Laboratory of Standards and Testing, Center for Biologics Evaluation and Research, Food and Drug Administration, USA) at 2 international units (IU) per ml. The slides were then incubated at least 1hr at 37 °C and 5 % CO_2 in humidity. Following incubation slides were washed three times in PBS PH 7.4 (Lonza, USA) and then air dried before flooding with fluorescein isothiocyanate (FITC) labeled mouse anti human IgG, (Sigma Aldrich, Germany) diluted 1:100 in PBS with 0.01% Evan's Blue (Sigma Aldrich, Germany). The slides were incubated at least 45 minutes at 37 °C and 5 % CO_2 in humidity before aspirating contents and washing three times in PBS. The plates were analyzed under a fluorescence microscope using a 450-490nm filter: FT 510nm: long pass (LP) 515nm and

10X eye piece: 10x Objective lens. The photographs were taken with a digital camera (Zeiss, USA) at the microscopy unit University of Pretoria, South Africa).

2.2.7 Culturing of recombinant vaccinia virus clones

A 25 cm^2 cell culture flask monolayer of Vero cells at cell density of 5 x 10^6 cells/ml was trypsinized and centrifuged for 15 minutes at (1500 X *g*) at room temperature. The supernatant was discarded and pellet resuspended in DMEM/F12 containing 2.5 % FCS. The total volume contained Vero cells at cell density of 2 x 10^7 cells/ml. The cells were incubated at 37 °C and 5 % CO_2 until 100 % confluent.

Virus stocks were freeze thawed at least 3 times with a brief centrifugation step to pellet cellular debris and supernatant used to infect Vero cells in 25 cm^2 cell culture flask at moi 0.02 to 1.0 plaque forming units (pfu) in 2 ml of serum free medium. After 2hr virus adsorption, complete medium (DMEM/F12 containing 2.5% FCS) was added to final volume of 10 ml and flask contents incubated 48-72 hrs until visible CPE. Virus was harvested from 25 cm^2 cell culture flasks by reducing cell culture medium to 5 ml; discarded 5 ml and harvested virus in remaining 5 ml by freeze-thawing 3 times. Virus material was collected in a 15 ml tube (Corning, USA); briefly centrifuged at 12 100 *g* on a benchtop centrifuge to pellet cellular debris then aliquoted and label appropriately. Aliquots were stored at -70 °C. Some virus supernatant was used to infect a 75 cm^2 cell culture flask of Vero cells monolayer as before (but in ~ 12 ml (or half of cell culture medium volume) (Sonification may be necessary to aid in harvesting of virus from virus factories). Contents of 75 cm^2 cell culture flask were incubated 48-72 hrs until 90 % CPE was visible and virus harvested as before. Harvested 75 cm^2 cell culture flask material was used as inoculum to infect ten 150 cm^2 cell culture flasks. Finally contents of 150 cm^2 cell culture flasks were harvested as before while retaining the supernatant and subsequently purified as described below.

2.2.8 Semi-purification of virus preparations

Contents of 150 cm^2 cell culture flask were harvested; pooled and transferred into 50 ml centrifuge tubes (Corning, USA) and subsequently centrifuged 10 minutes at 1500 *g*, 4 °C. The supernatant was collected and pooled into polycarbonate oakridge tubes (Oakridge,

USA) followed by injection with 40 % sucrose (Sigma Aldrich, Germany) solution (in 1mM Tris, pH 9.8) at the bottom of the tubes (about 1 ml of sucrose per tube). Contents were centrifuged 2 hrs at 19 000 X *g* at 4 °C after which the supernatant was discarded and pellet resuspended in 1ml DMEM/F12 (without FCS and antibiotics). The purified content was stored at -70 ºC.

2.2.9 Titration of virus preparations

2.2.9.1 In vitro plaque assay

Twenty-four hours prior to titration, a Vero cell suspension containing 10^5 cells/ml was prepared in DMEM/F12 supplemented with 10 % FCS (heat inactivated), 2mM glutamine and 1% of penicillin (100 units/ml)/streptomycin (100 µg/ml) amphotericin B (0.25 µg/ml) (Lonza). Briefly a 25 cm^2 cell culture flask monolayer was trypsinized with 1ml trypsin (Lonza) and incubated at room temperature 5-10 minutes to allow monolayer to detach. 8 ml DMEM/F12 containing 10 % FCS was added and contents pipette to a homogenous solution. Two hundred micro liters of cells was pipetted into 24 well plates (Nalge Nunc, USA) and volume made up to1ml with DMEM/F12 10 % FCS followed by 24hrs incubation at 37 ºC and 5 % CO_2 until 80 % confluent.

Virus was serially diluted in DMEM/F12 (without FCS) from 10^{-1} to 10^{-10}. Hundred micro liters of virus inoculum was added to each well (each dilution was done in duplicate) and allowed at least 2 hrs for virus to adsorb after which more media (900 µl) was added and contents subsequently incubated for 48 hours. Cells were examined under a light microscope for plaque formation and plaques counted to calculate the plaque forming units per ml (pfu/ml).

$$\frac{\text{(Number plaques)}}{\text{(Total volume in individual well)}} \times \text{Dilution factor} = \text{pfu/ml}$$

2.3. Results

2.3.1 Generation and culturing of homogenous recombinant vaccinia virus Copenhagen clones

Recombinant MokG, MokPV and PVPV were generated by transfection of vaccinia virus Copenhagen infected Vero cells with plasmid containing respective glycoprotein constructs. Subsequently recombinant viruses were analyzed by plaque assay for chimeric PVPV or for transient expression of EGFP for chimeric MokPV and homologous MokG. Recombinant virus was grown under MPA selection at least five times and homogeneity of recombinants monitored by PCR with vac TKr and vac TKl primers that bind regions flanking TK region of vaccinia virus. The recombinants yielded a band of approximately 3500 bp for PVPV and 4300 bp with MokG and MokPV whereas wild type vaccinia virus Copenhagen genomic DNA yielded a 300 bp amplicon (figures 2.1 and 2.2 and 2.3). Upon confirming homogeneity of recombinant viruses as to contain no parental wild type background, selection pressure was lifted.

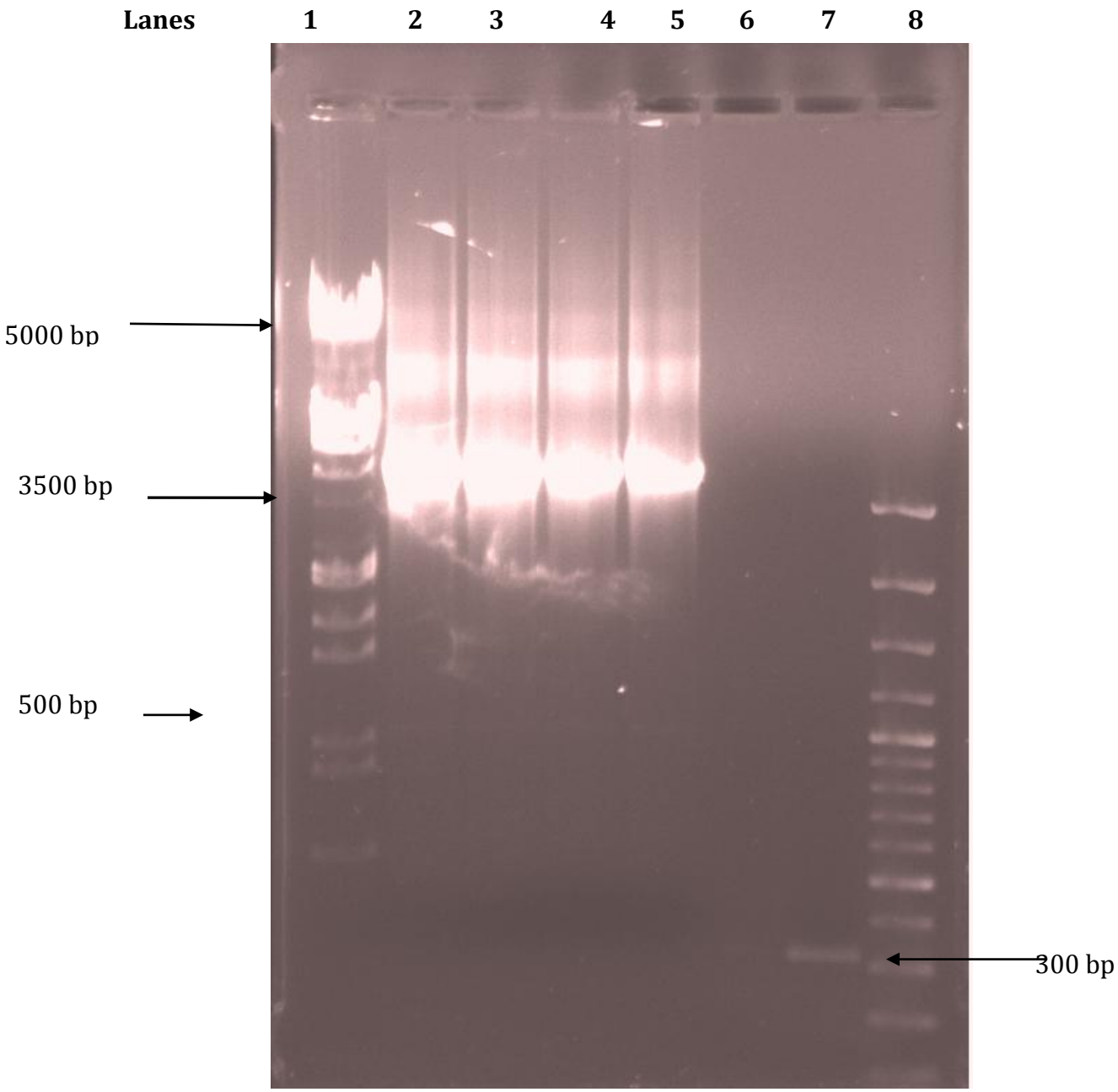

Figure 2.1: PCR amplicons obtained with VacTKl and VacTKr primers that anneal in regions flanking the TK region of vaccinia virus Copenhagen genome. In lane (1) 1Kb ladder DNA marker *Eco*R I/*Hin*d III (Promega) and lane (8) 100 bp marker (Promega); (2-5 Recombinant PVPV vaccinia Copenhagen vaccine virus); (6) negative control-nuclease free H_2O (7) Vaccinia virus genomic DNA

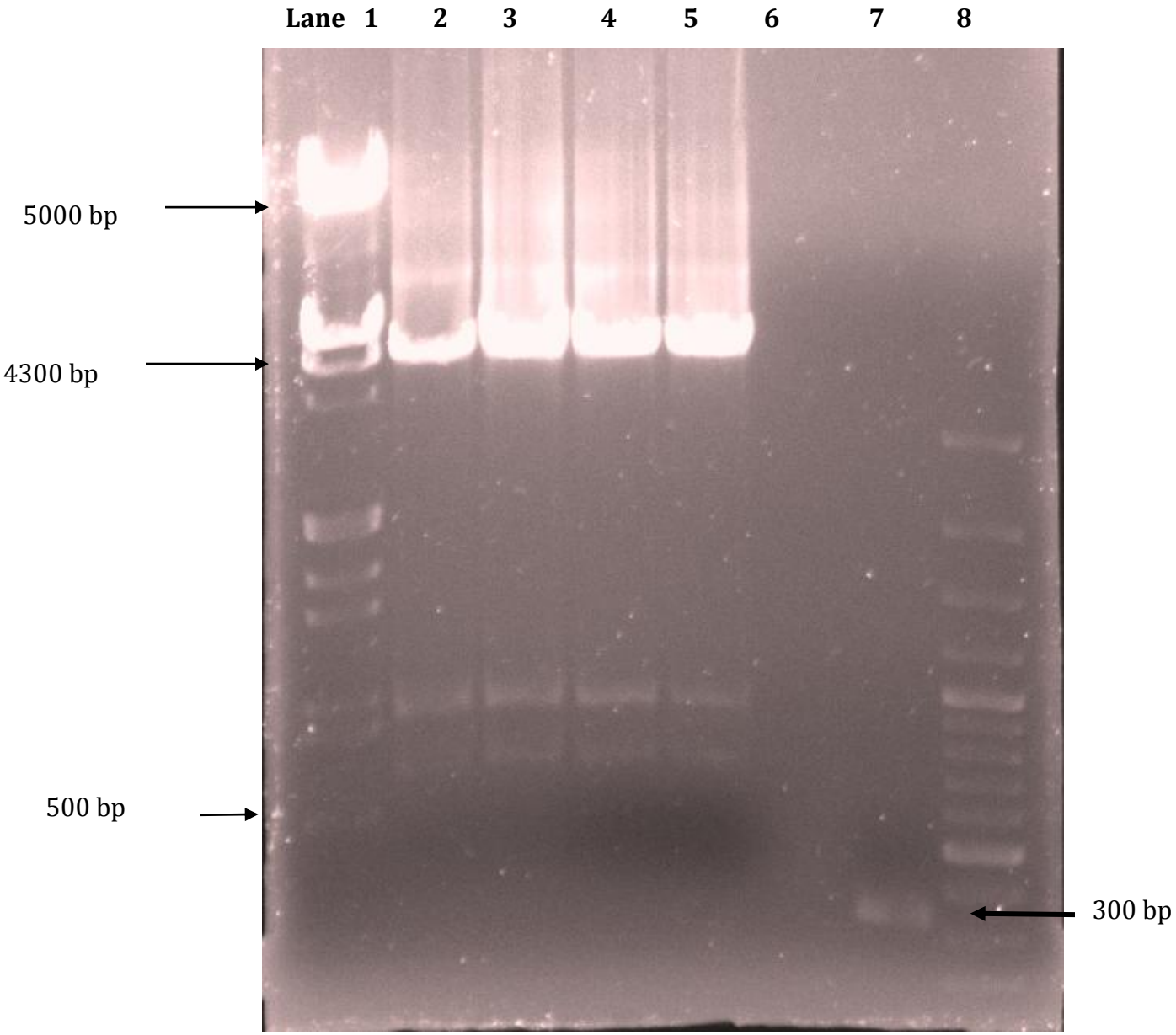

Figure 2.2: PCR amplicons obtained with VacTKl and VacTKr primers that anneal in regions flanking the TK region of vaccinia virus Copenhagen genome. In lane (1) 1Kb ladder DNA marker *Eco*R I/*Hin* dIII (Promega) and lane (8) 100 bp marker (Promega); (2-5 Recombinant MokPV Vaccinia Copenhagen vaccine virus); (6) negative control-nuclease free H_2O (7) Vaccinia virus genomic DNA

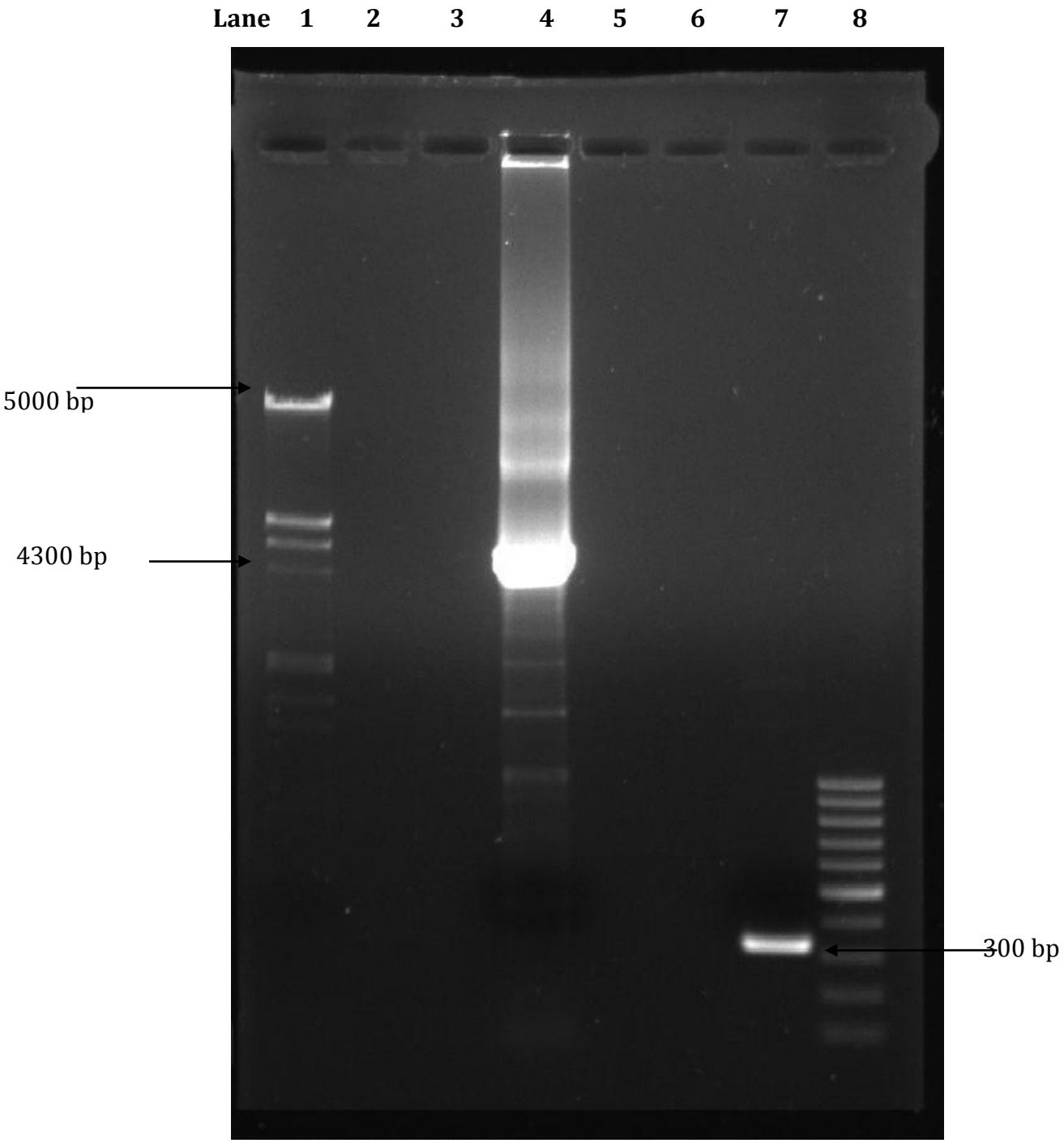

Figure 2.3: PCR amplicons obtained with VacTKl and VacTKr primers that anneal in regions flanking the TK region of vaccinia virus Copenhagen genome. In lane (1) 1Kb ladder DNA marker *Eco*R I/*Hin*dIII (Promega) and lane (8) 100 bp marker (Promega); (4 Recombinant MoKG Vaccinia Copenhagen vaccine virus); (6) negative control-nuclease free H_2O (7) Vaccinia virus genomic DNA

2.3.2 Sequencing of PCR amplicons

The integrity of homologous recombination sites within vaccinia virus Copenhagen vector as well as position of promoters within the expression cassette was verified by sequencing using vac TKr and vac TKl primers. The resulting sequence information revealed an ATG start codon at the beginning of the respective glycoprotein sequences (MOKG, MOKPV, PVPV). In addition, a 230 bp p7.5 Vaccinia late/early promoter was confirmed to be in precise orientation upstream of a 845 bp *E.coli* gpt site and the respective glycoprotein sequences. In all three cases these gene cassettes were flanked by approximately 180 bp sequences that were remnant of the thymidine kinase gene (from homologous recombination in the generation of recombinant viruses). (See appendix A).

2.3.3 Expression analysis

Expression of glycoproteins by recombinant viruses was confirmed by indirect immunofluorescence analysis. The SRIG was used as a primary antibody and FITC labeled anti-human IgG used as a secondary detecting antibody. Cells infected with CVS and MOKV as well as a negative control (only cells) were included. The recombinant constructs gave a strong signal comparable to the positive controls (figure 2.4a-d). On the contrary no signal was detected the negative control (figure 2.4e).

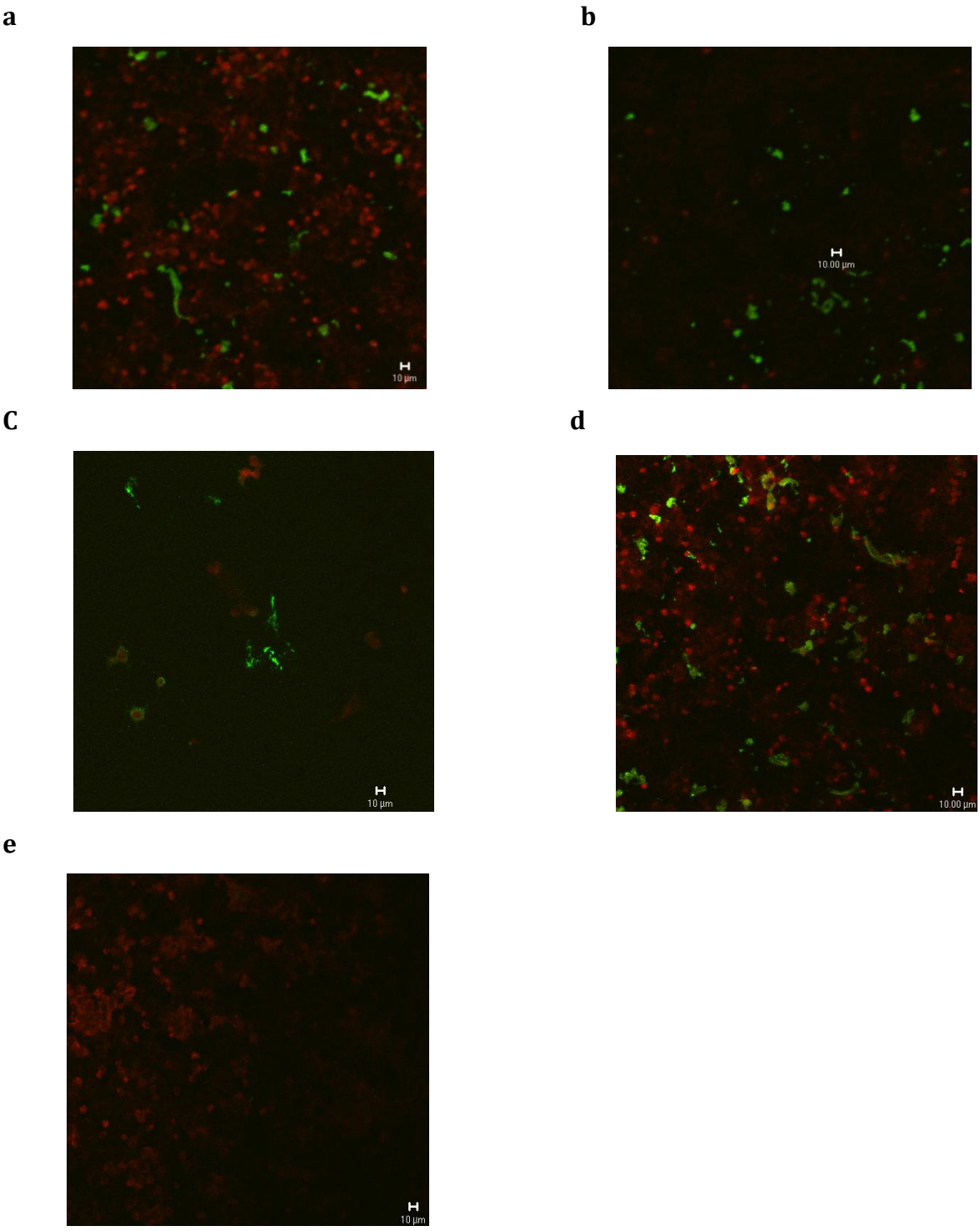

Figure 2.4: Indirect immunofluorescence assay of chimeric lyssaviruses glycoprotein in Vero cell culture. (a) Vero cells infected with CVS, (b) Vero cells infected with MOKV, (c) MokPV recombinant virus (d) PVPV recombinant virus (e) negative control (uninfected cells)

2.3.4 Culturing of recombinant vaccinia virus clones, semi-purification and titration

Large amounts of recombinant were achieved by growing viruses in larger surface area flasks. Contents of respective recombinants were pooled and purified in a sucrose gradient. In vitro plaque assays were used to determine titers of recombinants and the titers were estimated at 5.5 x 10^{10}pfu/ml (calculations not shown).

2.4. Discussion

The choice of vaccine vector is critical to maintain stable expression of antigen. In the current study vaccinia virus Copenhagen strain was used due to its large genome and capacity to accommodate a large number of foreign genes (Smith and Moss, 1983). The glycoprotein construct was inserted within vaccinia virus Copenhagen's TK gene as studies have shown TK region can be used to generate recombinant virus with distinct phenotype on TK- 143 cells (Chakrabarti *et al.*, 1985) and TK gene is non essential in virus growth in cell culture (Dubbs *et al.*, 1964). Disruption of TK gene by insertional inactivation by foreign gene has been shown to reduce virulence (Buller *et al.*, 1985). Growing of recombinant virus in MPA inhibited growth by stopping nucleotide synthesis. Inclusion of the *E.coli* gpt gene limited growth of vaccinia virus wild type on Vero cells and selected for only recombinant vaccinia virus carrying *E.coli* gpt gene.

Sequencing confirmed homologous recombination within vaccinia virus TK region. The integrity of the expression cassette was also verified and revealed the p7.5K promoter was maintained upstream *E. coli* gpt and glycoprotein. Early class promoters allow expression to occur before cytopathic effects become extensive and are therefore preferable to elicit cytotoxic T-lymphocytes responses within a host. The CTL are capable of acting as both helper T cells (CD4) and cytotoxic T cells (CD8). On the other hand late promoters permit expression of large amounts of foreign proteins (Falkner and Moss, 1988). Although the complete expression cassette was not sequenced to establish precise position of EGFP, transient expression of this protein was validated by immunofluorescence microscopy.

Recombinant vaccinia virus was shown to stably express the RABV and MOKV glycoproteins which were recognized by human rabies immunoglobulin (HRIG) conjugated to FITC labeled anti human IgG. The Evans Blue dye was used as a counter stain to minimize background non specific binding by staining non-infected cells red as in the case

of the negative control. The MOKV and RABV CVS strain positive controls are shown as cells stained completely green as the HRIG is a polyclonal raised mainly against nucleoprotein as well as to some level the glycoprotein. The MoKPV and PVPV recombinant viruses only stain green at periphery because vaccinia virus expresses the glycoprotein foreign gene construct at the plasma cell membrane. This implies the glycoprotein was processed to include appropriate glycosylation that generate a functional glycoprotein. The RABV glycoprotein by itself has been shown to be an efficacious vaccine (Kieny *et al.*, 1984; Prehaud *et al.*, 1989; Xiang *et al.*, 1994). Based on this finding, the recombinant virus was subsequently grown to high titers that would potentially induce an immune response in vivo.

The decision on when to harvest virus infected cells is important as it will determine the amount of virus progeny. Sufficient progeny virus is usually obtained late in an infection, example 48hrs post infection (p.i), as it allows maximum DNA replication which, combined with physical parameters like a large surface area of culture vessel, enable high virus titers. If infected cells are harvested after completion of an infectious cycle, most of extracellular enveloped virus (EEV) progeny is released into culture media – while the intracellular mature virus (IMV) is still in the cytoplasm. IMV are naturally released after cell death (72-96 hrs p.i). IMV constitutes most infectious progeny because it contains a small number of membranes, thus making uncoating during release of viral core much easier and is also more resistant to physical stress like freeze-thawing. Cells harvested before cell death were freeze-thawed at least three times to release IMV. In contrast, EEV are responsible for long range spread of virus which is characterized by formation of large plaques. The high recombinant virus titers obtained (up to 5 x 10^{10}pfu/ml) can be explained by presence of both infectious viruses at high concentrations.

Chapter 3

Use of a mouse model to evaluate the immune responses induced by recombinant vaccinia virus expressing different lyssavirus glycoprotein genes

3.1 Introduction

Vaccine potency is a key factor in determining the effectiveness of a vaccine. This is usually a reflection of the antigen content of a vaccine batch which in turn correlates to the titer of antibodies induced following inoculation. Several studies have focused on vaccine cross-protection within the *Lyssavirus* genus. In one such study the glycoprotein of MOKV was expressed in the baculovirus system (Tordo *et al*., 1993). In that study the protein expressed proved immunogenic, and protected against lethal MOKV challenge in a mouse model. However protection was not conferred against lethal challenge with RABV. In a different study MOKV glycoprotein gene expressed in different DNA vaccine carriers protected against MOKV virus challenge upon booster administration but failed to protect against a heterologous RABV challenge (Nel *et al.,* 2003). In a different study chimeric lyssavirus glycoprotein genes carrying critical glycoprotein antigenic sites II and III from respectively MOKV and RABV were expressed in a DNA vaccine model (Jallet *et al*., 1999). These constructs protected against both the viruses in a mouse model (Bahloul *et al*., 1998). The sera collected from animals immunized with the chimeric constructs were tested for cross reactivity against an array of lyssaviruses (Bahloul *et al.,* 1998; Desmézières *et al*., 1999). These sera significantly cross neutralized LBV, European Bat lyssavirus-2, but only partially cross neutralized Duvenhage and European bat lyssavirus-1 viruses (Bahloul *et al*., 1998). In another study the efficacy of recombinant vaccinia viruses expressing full-length lyssavirus glycoprotein genes have been shown (Weyer, 2006). Patterns of cross-protection were similar to that found by Bahloul *et al.,* 1998.

The purpose of the current investigation was to add to studies discussed above and investigated the immune responses elicited in mice, upon the administration of recombinant vaccinia virus-based vaccines expressing different lyssavirus glycoprotein genes and one chimeric lyssavirus glycoprotein gene. Mouse models are relatively simple and practical to use, and has therefore been extensively used and proven useful as a first line model in evaluation of various biological properties of viruses and of immune reponses upon infection of vaccination. At this point in the current study, the evaluation of the immunity induced by the vaccine constructs was based on

RABV VNAbs. It is also well proven that the level of VNAbs can be directly correlated to protection against infection (Templeton *et al*., 1986; Moore and Hanlon, 2010).

Serological methods that can be used to detect and quantify VNAbs, are based on inhibition of RABV infection in cell culture (Atanasiu, 1973). While the WHO previously recommended the *in vivo* virus neutralizing test (VNT) (in mice), the VNT is limited in its applicability since its time consuming, relatively costly and, has in recent times been replaced by the *in vitro* rapid fluorescent focus inhibition test (RFFIT) for determination of VNAbs to RABV. The RFFIT method is usually followed as described in (Smith *et al*., 1973) and involves antibody detection as indicated by a reduction in number of fluorescing foci of a virus infected cell culture. In this assay the serum neutralization end-point titer is defined as the dilution factor that neutralizes 50 % to 100 % of the challenge inoculum. Fluorescent antibody virus neutralization (FAVN) is a modification of RFFIT carried out in a 96 well microtiter plate. Zalan first described the FAVN in 1979 (Cliquet *et al.,* 1998; 2000). Studies have confirmed 86.6% correspondence between VNT and FAVN and a 95% correspondence obtained with RFFIT and FAVN (Ondiejkova *et al*., 2002). The same study found no significant difference with regard to sensitivity, specificity and reproducibility between these methods. Based on these suggestions RFFIT was used in our study to analyze humoral response to rabies.

The vaccine efficacy is reflected in its ability to cause seroconversion in mice, with respect to rabies this is a measure of VNAbs and is expressed in international units/ml (IU/ml) (WHO, 2000). A level of 0·5 IU/ml is as an arbitrarily defined threshold indicative of protection against RABV infection. The VNAb titers as calculated from RFFIT are usually converted to IU/ml to quantify the extent of seroconversion.

This chapter describes the evaluation and comparison of the protection afforded by two chimeric lyssavirus glycoprotein genes (MokPV and PVPV) as well as a homologous MOKV glycoprotein gene (MokG), in a mouse model.

3.2 Materials and methods

3.2.1 Animals used in the study

Ethical approval was obtained from the Animal Ethics Committee of the National Health Laboratory Services (AEC-NHLS) South Africa (certificate number 115/09/2). Six week old female BALB/c mice (supplied by South African vaccine producers) were used in this study. Animal housing and handling was as approved by AEC-NHLS.

3.2.2 Immunization of mice

A total of 45 BALB/c mice were used in this study, mice were subdivided into groups of 15 mice with each group receiving one of three recombinant vaccines (namely MokPV recombinant vaccine; PVPV recombinant vaccine and MokG recombinant vaccine). The 15 mice within a vaccine group were further subdivided in three groups of 5 mice/cage. The mice are hereafter referred to individually as mice 1 to 15. All 15 mice were immunized on day 0 with one of three recombinant vaccines. On day 10 (mice 1-5) were sacrificed and blood collected, on day 14 the remainder of mice (6-15) were divided into two groups of 5 with first group receiving no booster dose (mice 6-10) and second group receiving a booster (11-15). Mice were kept until day 28 when both the two groups were sacrificed and blood collected. A control group (n = 5) consisting of non-immunized mice was also included. This was a preliminary study and only five mice were used per group. Based on previous studies on vaccine efficacy Weyer, 2007, this number was considered sufficient to quantify the VNAbs. The details of the procedures undertaken are illustrated in table 3.1.

Table 3.1 Immunization schedule with different recombinant vaccines

	MOKPV recombinant vaccine		**MOKG recombinant vaccine**		**PVPV recombinant vaccine**	
Day 0	Immunized i.m mice with 10^7pfu in 50 µl n = 15		Immunized mice i.m with 10^7pfu in 50 µl n = 15		Immunized mice i.m with 10^7pfu in 50 µl n = 15	
Day 10	Blood was collected from 5 mice immunized on day 0		Blood was collected from 5 mice immunized on day 0		Blood was collected from 5 mice immunized on day 0	
Day 14	Boost mice with 10^5 pfu in 50 µl n = 5	No boost n = 5	Boost mice with 10^5 pfu in 50 µl n = 5	No boost n = 5	Boost mice with 10^5 pfu in 50 µl n = 5	No boost n = 5
Day 28	Blood collected n = 5	Blood collected n = 5	Blood collected n = 5	Blood collected n = 5	Blood collected n = 5	Blood collected n = 5

The vaccine doses were administered intramuscularly using a 1 cc tuberculin syringe with 26-gauge needle in the right quadriceps muscle. Mice were anaesthetized using Ketamine (Anaket-V) and Xilasien (Chanazine) blood was then collected using a sterile 1 cc tuberculin syringe with a 21 gauge needle (BD microtainer™ USA). The blood was collected into Microtainer® serum separation tubes with SST™ (Becton Dickinson and Company, USA) as suggested by the manufacturer. Sera was separated by centrifugation of serum separation tubes at 14 800 *g* for 10 minutes and stored at – 20 °C until analysis. Collected serum was used to analyze the antibody response by different vaccines in presence or absence of booster doses.

3.2.3 Rapid fluorescent focus inhibition test (RFFIT)

The virus-neutralizing antibodies (VNAbs) in mice sera were determined as described by Kuzmin *et al.,* 2008, with some modifications. This is a modification of the rapid fluorescent focus inhibition test (RFFIT) (Smith *et al*., 1996).

3.2.3.1 Preparation of RFFITs

The tests were prepared in eight-well (6-mm) Teflon-coated glass slides (Cel-line/thermo scientific). CVS, LBV/06 and MOKV/252 were used as challenge virus to study immune response induced in mice after immunization with chimeric MOKPV, PVPV and homologous MokG vaccines. The recombinant and challenge viruses were grown in MNA cells with DMEM/F12. The media's pH of 7.4 along with supplemented vitamins and amino acids are necessary for growth of MNA cells. The serum was first heat inactivated for 30 minutes at 56 °C to inactivate compliment. Serum dilutions were set in eight serial 2.5-fold dilutions, In brief, 3.5 µl of serum was mixed in the first chamber with 14 µl of DMEM/F12 making a 1:10 dilution. Further, 5 µl of this mixture was transferred to next chamber and mixed with 7.5 µl of DMEM/F12, this was repeated until the last well with 5 µl in the last well removed.

Thereafter 12.5 µl of challenge viral inoculum diluted in DMEM/F12 to yield 50 % focus forming dose (FFD_{50})/0.1ml was added to each chamber and the slides were incubated in a humidity chamber for 90 minutes at 37 °C in the presence of 5 % CO_2. After the incubation, a fully confluent mouse neuroblastoma (MNA) cell monolayer in 75 cm^2 flask was trypsinized and resuspended in 7.5ml DMEM/F12 (a fully confluent 75 cm^2 contains 15 x 10^6, resuspending it in 7.5ml should have 2 x 10^6 cells/ml). 25 µl of the MNA cells containing (2 x 10^6 cells/ml) were added into each chamber. Slides were incubated at the same conditions for 20 to 44 hrs before acetone fixation and staining.

The controls included a virus titration control prepared by setting up a back titration of the challenge viruses (CVS, LBV and MOKV) in a 10 fold serial dilution (i.e. 50 FFD_{50}, 5 FFD_{50} and 0.5 FFD_{50}), a reference sera control prepared in four 2.5-fold dilution (1:10-1:160) and one well was left uninfected to serve as a cell culture control. Briefly 14 µl of DMEM/F12 was added to the first chamber of reference sera control and 7.5 µl of DMEM/F12 was added to remaining chambers of reference sera (1:25-1:160). Three and a half micro liter of standard rabies immunoglobulin (Laboratory of Standards and Testing, Center for Biologics Evaluation and Research, Food and Drug Administration, USA) at 2 international units (IU) per ml was added to the first chamber of the control slide, and serially diluted by transferring 5 µl in four, 2.5-fold dilutions (i.e. 1:10, 1:25, 1:165 and 1:160). Twelve and a half micro liter of virus containing 50 FFD_{50}/0.1ml was added to all chambers of reference

sera dilution while 10 µl of 0.5 FFD_{50}, 5 FFD_{50} and 50 FFD_{50} stock virus was added to respective chambers. No virus was added to the cell control. The dilutions were incubated at 37 °C and 5 % CO_2 for 90 minutes. Following incubation MNA cells were added to the reactions. A suspension of cells was prepared as before, 25 µl of the cell suspension was added to each chamber of the tests. The preparations were returned to the incubator for another 24 hours.

3.2.3.2 Fixation, staining and reading of the tests

Following incubation, cell culture supernatants were discarded and the slides dip-rinsed in PBS (pH 7.3). The slides were then transferred to ice-cold acetone for 30 minutes. After the incubation slides were washed twice in PBS and air dried. FITC labeled anti-lyssavirus conjugate (Sigma Aldrich, Germany) was prepared at working dilutions (1:700). Approximately 20 µl of conjugate was pipetted onto each chamber. The slides were incubated at 37 °C for 45 minutes. After incubation slides were dip-rinsed twice in PBS for 10 minutes. The slides were then air dried before reading under a fluorescent microscope (Axioskop, Zeiss). The slides were read at 100 times magnification (10X eye piece: 10x objective lens), 10 microscope fields per chamber were observed and noted. If a reduction or absence of fluorescence was observed, the serum sample was subjected to additional titration. The 50 % end point neutralizing titers were calculated according to the Spearman Kaber formula (Aubert, 1996).

3.2.4 Statistical analysis

Statistical analysis of the variance between vaccine groups was calculated using the program statistica version 9.0 (licensed to the University of Pretoria). An analysis of variance (ANOVA) was perfumed as described in (ANOVA; Zar, 1996). The distribution of data (antibody titer) within vaccine groups determined the parameters to be used. A one way ANOVA was performed in case of a normal distribution and F and P values reported. Where applicable a square root transformation of non parametric data was run to normalize data and ANOVA performed. P value less than 0.001 with this test indicated a statistically significant difference. A Mann-Whitney U Test was performed in the case of non-normally distributed data and Z and P values reported. P value less than 0.05 with this test indicated a statistically significant difference (see appendix C).

3.3. Results

3.3.1 Neutralizing antibody response to various lyssaviruses

3.3.1.1 Neutralizing antibody response to RABV

The neutralizing antibody titers to RABV after immunization with the three different recombinant vaccine viruses is reported in table 3.2. Briefly all animals immunized with a single dose of the PVPV recombinant vaccine construct, seroconverted (Table 3.2, figures 3.1 and 3.2). However, animals immunized and boosted with the PVPV recombinant vaccine construct did not only seroconvert but the titre of VNAbs measured were significantly higher ($p< 0.001$) in animals that received a booster dose of the vaccine construct (figure 3.2). In the case of the MokPV recombinant vaccine construct, a single dose was not sufficient and only those animals immunized and boosted did in fact seroconvert (figure 3.3). Even so, the VNAbs measured for this construct, were significantly lower ($p < 0.05$) than those obtained with PVPV. A variation in the neutralizing antibody titers was noted within the experimental groups that received vaccine. The variation was significant in the case of animals immunized with a single dose of the PVPV recombinant vaccine construct (figure 3.1). It is noteworthy that VNAbs were comparable at days 10 and 28 between animals that only received a single dose of PVPV recombinant vaccine

Table 3.2: Neutralizing antibody titers[a] to RABV upon immunizing mice with different recombinant vaccinia virus vaccines. Titers are noted for day 0 (naïve sera), day 10 (Ten days after primary vaccination) and day 28 (14 days after booster vaccinations). Titers reported as means as determined with RFFIT.

Vaccine group	Day 0	Day 10	Day 28
MokPV	0.0	0.0	82.4
PVPV	0.0	542.6	1252.8
MokG	0.0	0.0	0.0

[a] geometric mean titers, reported as 10^ log (50 % End-point dilution) as determined with RFFIT

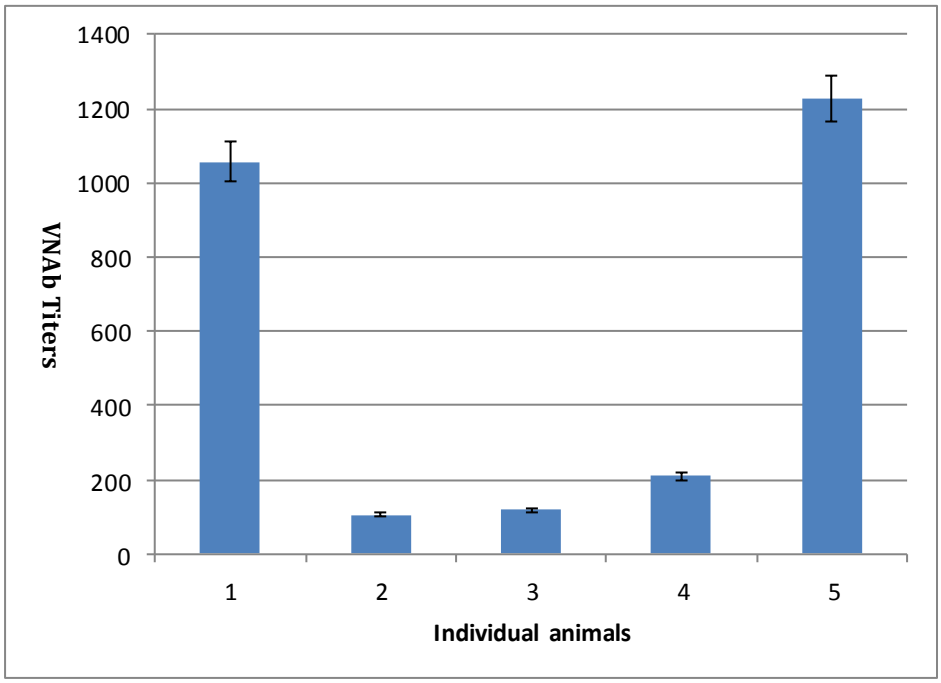

Figure 3.1: VNAb titers elicited (day10), after only primary immunization with PVPV recombinant vaccine which neutralized RABV (CVS-11) (bars indicate standard error).

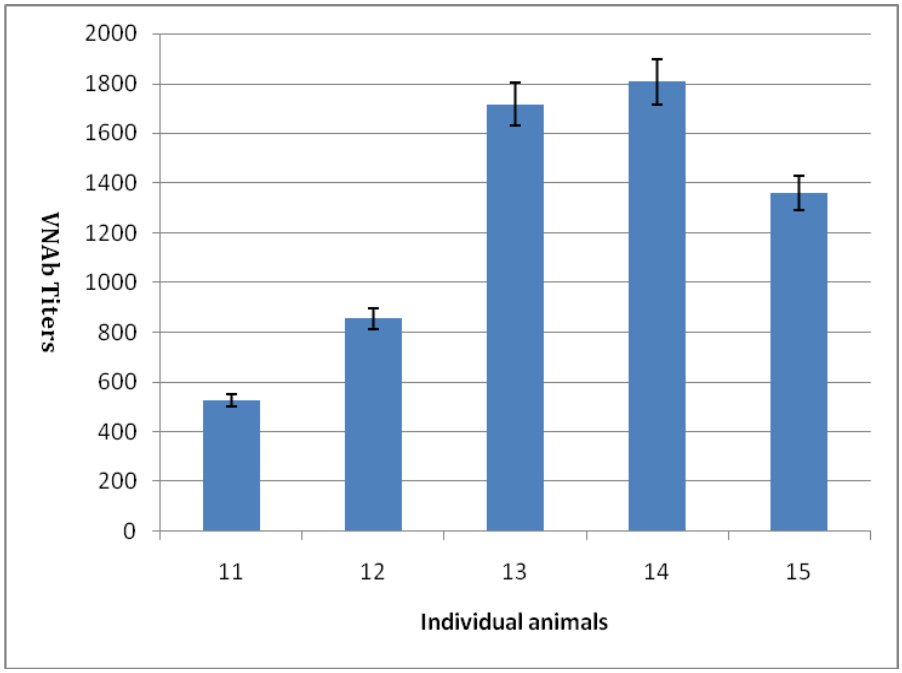

Figure 3.2: VNAb titers elicited (day 28), after immunizing and boosting with PVPV recombinant vaccine which neutralized RABV (CVS-11) (bars indicate standard error).

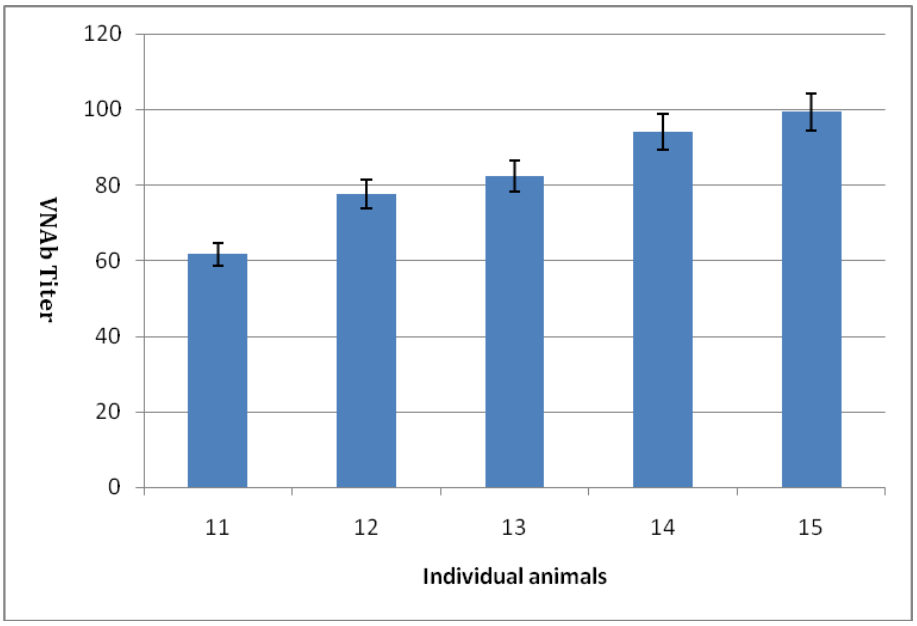

Figure 3.3: VNAb titers elicited (day 28), after immunizing and boosting with MokPV recombinant vaccine which neutralized RABV (CVS-11) (bars indicate standard error).

3.3.1.2 Neutralizing antibody response to MOKV

Table 3.3 documents the neutralizing antibody titers to MOKV upon immunizing with the different recombinant vaccine viruses. Briefly all animals immunized with a single dose of

the recombinant MokG vaccine construct seroconverted (Table 3.3, figures 3.4 and 3.5). Moreover animals immunized and boosted with the MokG recombinant vaccines not only seroconverted but had significantly higher (P< 0.001) titers of VNAbs than animals that only received a single dose of the vaccine. Moreover VNAbs were comparable at days 10 and 28 between animals that only received a single dose of MokG recombinant vaccine. In contrast the MokPV recombinant vaccine construct did not induce adequate VNAbs with a single dose and only those animals immunized and boosted seroconverted (figure 3.6). Still, the VNAbs measured with this construct, were significantly lower ($p < 0.05$) than those obtained with MokG.

Table 3.3: Neutralizing antibody titers[a] to MOKV after immunizing mice with different recombinant vaccinia virus vaccines. The titers are noted for day 0 (naïve sera), day 10 (Ten days after primary vaccination) and day 28 (14 days after booster vaccinations) Titers reported as means as determined with RFFIT.

Vaccine group	Day 0	Day 10	Day 28
MokPV	0.0	0.0	116.8
MokG	0.0	258.2	1662.8
PVPV	0.0	0.0	0.0

[a] geometric mean titers reported as $10^{\wedge \log(50\ \%\ \text{End-point dilution})}$ as determined with RFFIT

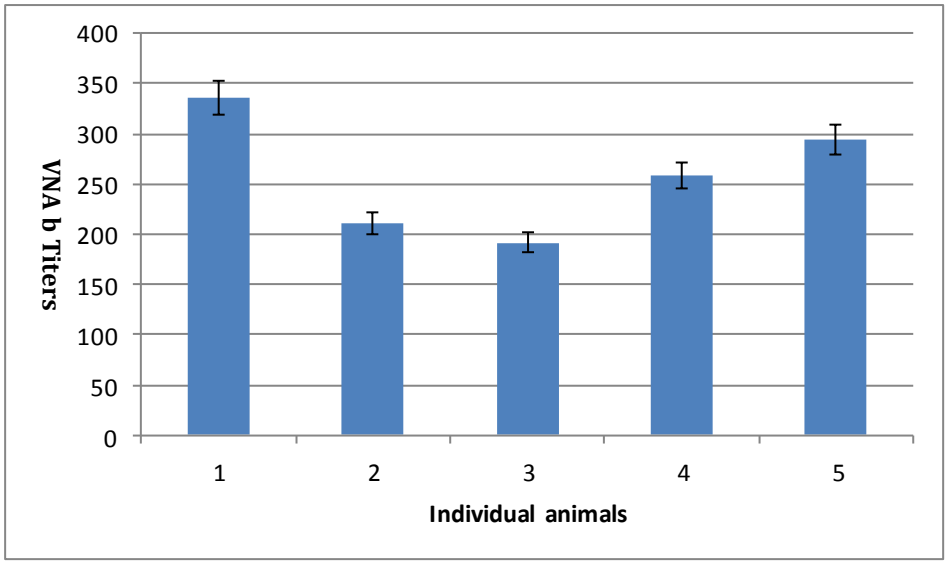

Figure 3.4: VNAb titers (day 10), elicited after only primary immunization with MokG recombinant vaccine which neutralized MOKV (bars indicate standard error).

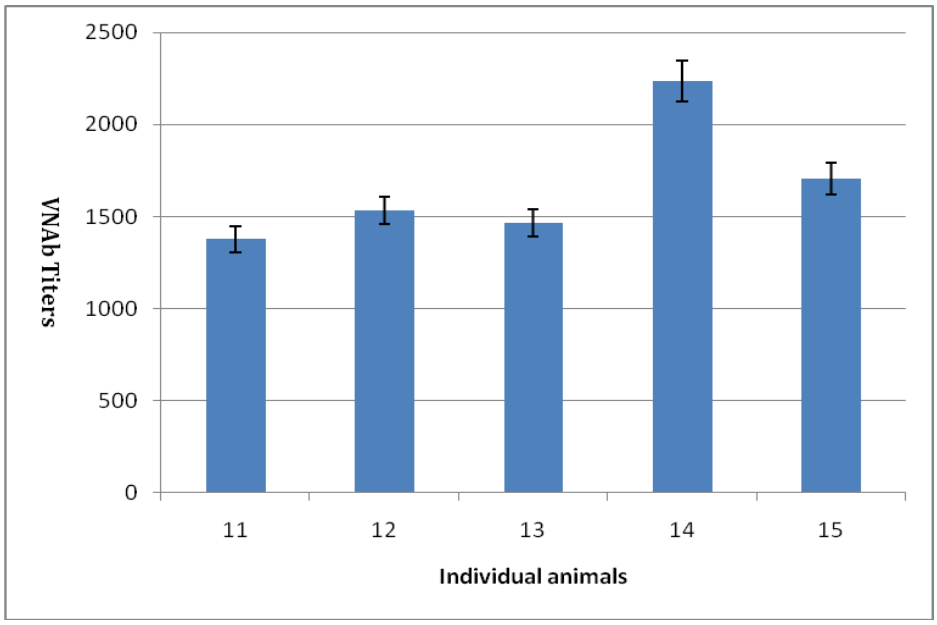

Figure 3.5: VNAb titers elicited (day 28), after immunizing and boosting with MokG recombinant vaccine which neutralized MOKV virus (bars indicate standard error).

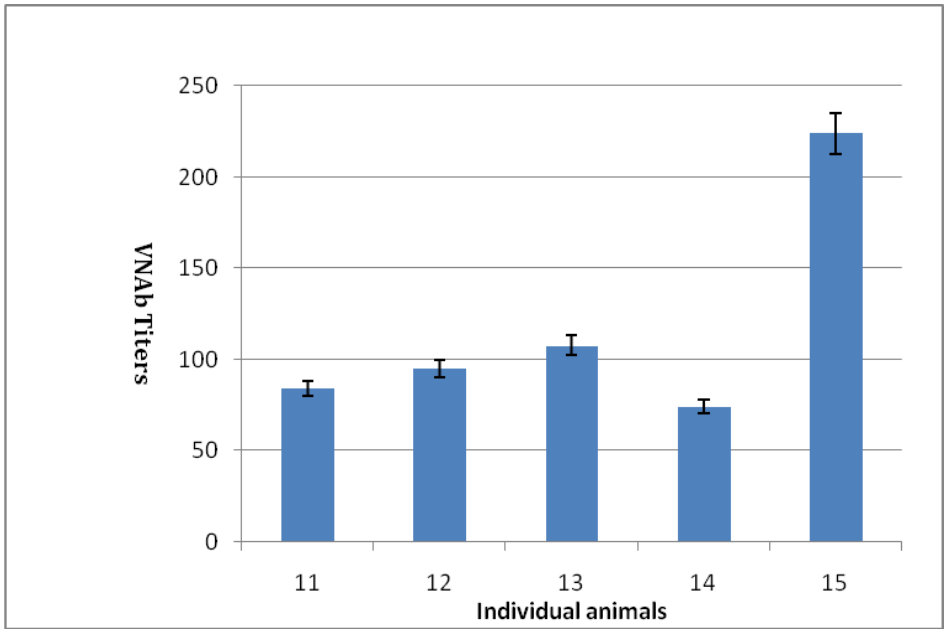

Figure 3.6: VNAb titers elicited (day 28), after immunizing and boosting with MokPV recombinant vaccine which neutralized MOKV (bars indicate standard error).

3.3.2 Cross-reactivity studies

Sera collected from animals that were immunized and boosted with the MokG recombinant vaccine construct, cross-neutralized LBV (figure 3.7). Sera collected from animals that were immunized and boosted with the MokPV recombinant vaccine construct also cross-neutralized LBV (figure 3.7 and figure 3.8) . In general, the MokPV recombinant vaccine induced significantly higher VNAbs against MOKV and LBV than against RABV (figure 3.8). Interestingly, the level of cross neutralization for sera from animals immunized with MokPV and those immunized with MokG, was similar (figure 3.7).Table 3.4 indicates the cross neutralizing antibody titers induced by various recombinant vaccines against LBV.

Table 3.4: Neutralizing antibody titers[a] to LBV after immunizing mice with different recombinant vaccinia virus vaccines. Titers are noted for day 0 (naïve sera), day 10 (Ten days after primary vaccination) and day 28 (14 days after booster vaccinations). Titers reported as means determined with RFFIT

Vaccine group	Day 0	Day 10	Day 28
MokPV	0.0	0.0	51.4
MokG	0.0	0.0	64.2
PVPV	0.0	0.0	0.0

[a] geometric mean titers reported as 10^ log (50 % End-point dilution) as determined with RFFIT

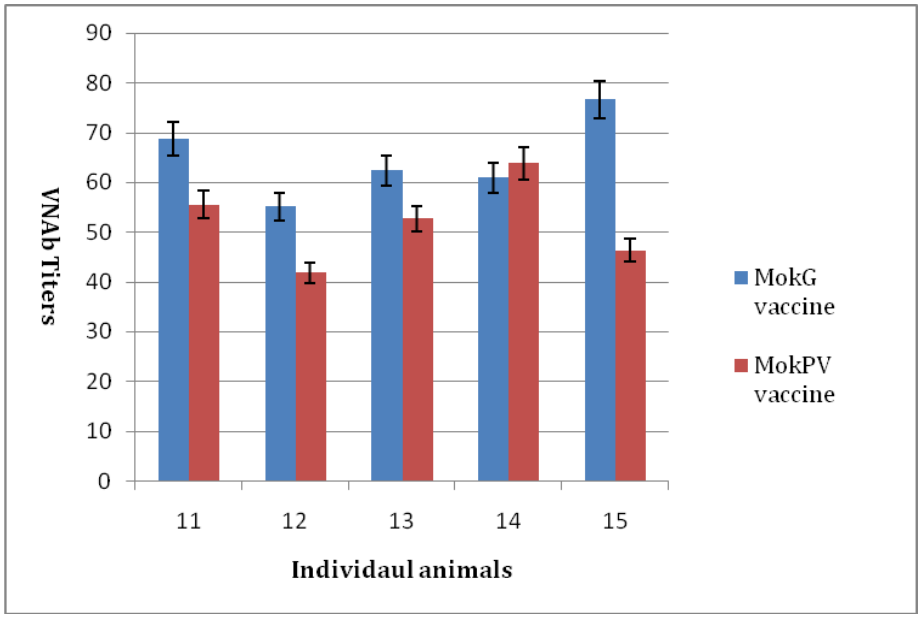

Figure 3.7: Comparison of the levels of VNAbs, elicited upon immunization with respectively MokG and MokPV recombinant vaccines, that also cross-neutralized LBV (bars indicate standard deviation between mean titers)

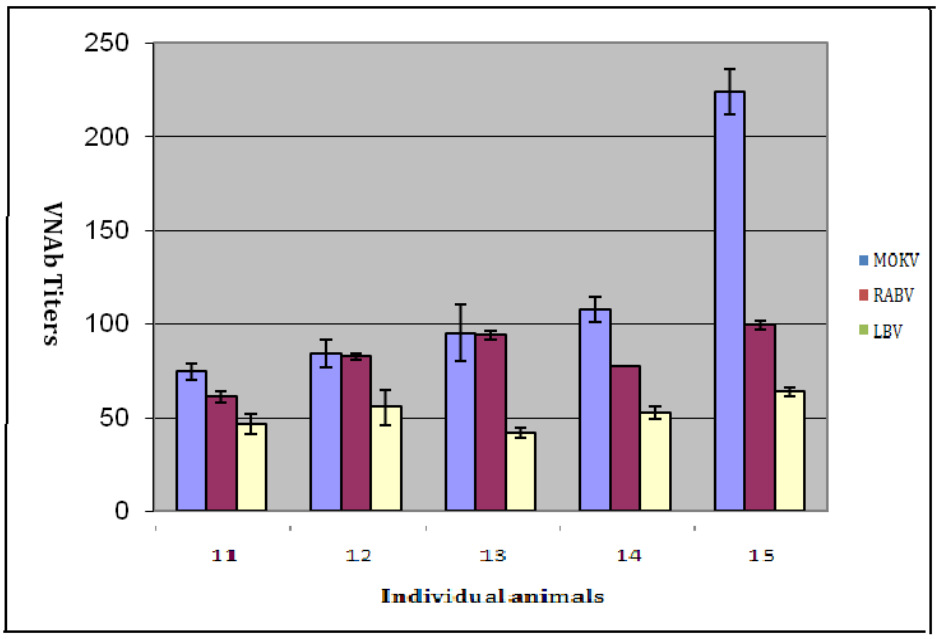

Figure 3.8: Comparison of the levels of VNAbs, elicited upon immunization with the MokPV recombinant vaccine which neutralized MOKV and RABV and also cross-neutralized LBV (bars indicate standard deviation between mean titers)

3.4 Discussion

The glycoprotein ectodomain of lyssaviruses has been documented to be most important in induction of VNAbs (Gaudin *et al*., 1992; Delagneau *et al.,* 1981), of particular interest is its two main antigenic sites II and III. This study provided a proof of concept that vaccinia virus vectors can effectively express, process and present not only homologous foreign lyssavirus glycoprotein genes, but indeed chimeric glycoprotein genes. Although no protective of VNAb is known for rabies-related lyssaviruses, it has been suggested that increased VNAbs titers (as elicited by a RABV-specific vaccine) are necessary for neutralization of the rabies-related lyssaviruses (Hanlon *et al*., 2005). In this regard a replicating vaccinia virus Copenhagen strain was employed in the anticipation that high neutralizing antibody titers will be achieved. The findings of the study clearly demonstrated that neutralization titers against a homologous challenge are highest if both site II and III of glycoprotein are present. This was illustrated with the MokG and PVPV recombinant vaccine constructs. The sera collected after immunization with these homologous vaccines contained high VNAb titers against respectively MOKV and RABV. A significant difference between boosted and non-boosted vaccine constructs was noted for

both recombinant vaccines, MokG and PVPV. In contrast, recombinant MokPV, expressing the chimeric glycoprotein gene, elicited a detectable VNAb titer only upon a booster dose administration. The titers were still significantly lower than those achieved with the homologous vaccines (MokG against MOKV and PVPV against RABV). This result suggests that both sites II and III are required to induce an early virus neutralization response against a homologous challenge and also points at a structural and subsequently an immunological dependence of sites II and III. The RABV glycoprotein ectodomain contains 14 conserved cysteine residues. All these 14 cysteine residues are conserved in the MOKV isolate (accession number U17064) used in chimeric vaccine construction, suggesting similar folding through disulfide bridges (Mebatsion *et al.,* 1994). Cysteine residues have been shown to be important in glycoprotein folding by molecular chaperones in the endoplasmic reticulum (Gaudin, 1997). The fact that the glycoprotein antigenic sites II and III fold with the same kinetics also suggests that these sites are very close to each other in the three-dimensional structure of the glycoprotein (Gaudin, 1997).

Despite lower VNab titres, the sera from mice immunized with MokPV recombinant vaccine nevertheless neutralized both MOKV and RABV. Similar protection patterns were reported with DNA vaccines (Jallet *et al*., 1999). Interestingly the neutralization achieved with MokPV vaccine against MOKV was significantly higher than that obtained against RABV (CVS-11). This finding could suggest a more important role of site II in the protection against homologous virus challenge. In support of these results are previous studies which have shown that RABV site II is recognized by approximately 72 % of mAbs raised against the glycoprotein while site III is recognized by only 24 % of the mAbs (Benmansour *et al.,* 1991; Lafay *et al*., 1996; Lafon *et al*., 1983). A comparison of the amino acid sequence of the glycoprotein used in the chimeric MokPV vaccine construct and that of MOKV isolate 252/97 used as challenge virus in the serological assays revealed the following. Glycoprotein antigenic site II cysteine residues 35 and 207 were conserved in both isolates. Furthermore the residue 198 was a conserved lysine in the MOKV 252/97 isolate but was substituted for a glutamine in the chimeric vaccine construct.

The cross-neutralization studies demonstrated that MOKV shares similar epitopes with LBV. Sera collected from mice immunized and boosted with either MokG or MokPV

recombinant vaccines cross-neutralized LBV as indicated by RFFIT. Interestingly, similar VNAb titers for LBV were reported with both MokG and MokPV recombinant vaccines. This again reinforces the suggestion that site II carries more important epitopes needed in cross-protection as it is the only major glycoprotein antigenic site common in MokG and MokPV recombinant vaccine constructs. An amino acid sequence comparison between site II of the MOKV used in the chimeric vaccine (accession number U170640) and site II of challenge virus (LBVSA2006) showed that the Cysteine residues 35 and 207 were conserved. Moreover the residue 198 was maintained as a lysine in the LBVSA2006 virus but was substituted for a glutamine in the vaccine construct.

Notably, the VNAb titers elicited by replicating vaccinia virus Copenhagen vaccine vector carrying chimeric MokPV or PVPV constructs as well as homologous MokG construct were significantly higher than those obtained with DNA vaccines expressing similar constructs (Bahloul *et al.*, 1998). This finding is of particular importance when considering vaccine candidates to employ in the control of rabies in Africa where the primary rabies vectors, domestic dogs, are mostly free roaming. This makes it difficult in case of a vaccine that requires a booster dose to attain sufficient VNAbs titers. The PVPV recombinant vaccine qualifies as a candidate in this respect as it was shown that it induced high VNAb titers with both boosted and non-boosted vaccines (respectively 157.518IU/ml and 72.5508IU/ml). Variation in VNAb titer was noticed within different vaccine groups, this could be due to a difference in response to foreign immunogen by different animals. Except for animals that received a single dose of the PVPV recombinant vaccine, these variations were not significant within most groups. Two mice that only received a primary vaccine dose of the PVPV recombinant vaccine had comparable virus neutralization titers to mice that received a booster dose, confirming the potential value of vaccinia virus-based vaccines as single dose biologic.

Chapter 4

Concluding remarks

Due to limited epidemiological data and lack of active surveillance, little is known regarding the rabies-related lyssaviruses. Nevertheless, the public health risk and potential burden should not be underestimated and a need to develop vaccines that confer cross-protection across the pan-lyssavirus spectrum has been the focus of recent studies. The choice of vector - a replicating vaccinia virus based vector demonstrated a benefit as both chimeric and homologous recombinant vaccine constructs elicited protective neutralizing antibody titers (above 0.5IU/ml recommended by WHO). This study was specifically aimed at the evaluation of the immunity induced by the recombinant and chimeric constructs as judged from VNAb responses. It is well proven that the level of VNAbs can be directly correlated to protection against infection, and in keeping with the purpose of the study, cellular immune responses were not evaluated, nor were animals sacrificed by fatal challenge.

A significant finding in the current study was that protection conferred by site II and III towards a homologous virus is not equal. Site II confers better protection against a homologous virus. Protection is highest when both sites II and III of homologous virus are present. This points out at a complex structural interaction between antibody epitopes on glycoprotein and various immune effector mechanisms. Structural folding of the glycoprotein reveals that different mAbs bind different sections of the glycoprotein stimulating different independent antibody isotypes with varying neutralizing activity (Schumacher *et al.*, 1989).

The results generated in our study complimented previous findings with DNA vaccines (Bahloul *et al.*, 1998) that vaccine constructs with both sites II/III of the same virus conferred higher protection against homologous challenge than vaccine constructs with MOKV site II and RABV site III. Nevertheless, despite lower VNAbs with chimeric MokPV, the vaccine neutralized all challenge viruses used in the study (MOKV, RABV and LBV). This highlights the compromise between high vaccine VNAbs and vaccine cross-reactive capacity. The supplementary findings with this study were that replicating vaccinia virus

Copenhagen had significantly higher VNAbs than preceding DNA vaccines. The importance of MOKV site II in protection of homologous virus and indeed in cross-neutralization of heterologous LBV was also demonstrated. Based on these findings it could be speculated that the MokPV chimeric construct would also cross protect the new SHIBV. Recent studies by Kuzmin *et al.,* 2010, showed that although there is no significant sequence conservation between SHIBV and phylogroup II lyssaviruses at positions 34–42 within the glycoprotein antigenic site II, another part of this antigenic site (positions 198–202) was identical between SHIBV and the LBV lineages C and B. In that study it was shown that the genetic distance between SHIBV and LBV, is in the same range as the distance between LBV and MOKV. On that note, West Caucasian bat virus is the most divergent of the *Lyssavirus* genus, Kuzmin *et al*., 2005 and therefore we do not anticipate our chimeric vaccine constructs to cross protect WCBV.

In this study, the potential of recombinant chimeric as well as full length glycoprotein vaccine constructs to induce VNAbs was demonstrated and the parenteral route of vaccine administration was chosen. The efficacy of these constructs in an oral vaccine administration can only be speculated. The vector backbone used in this study –vaccinia virus Copenhagen strain has been successfully used as an oral vaccine (Pastoret and Brochier, 1996). It can be anticipated that these constructs would induce some immunity via the oral mucosal route in wildlife. However these vaccines could have a limitation in humans due to the possibility of an anti-vaccinia immunity. Some studies have shown that an anti-vaccinia immunity could be prevalent in populations that have been exposed to the vaccinia virus during smallpox vaccination (Belyakov *et al*., 1999).

To improve on immune protection and cross-protection, future studies could focus on enhancement of both adaptive and innate immune responses by employing one of the following developments. Firstly, the limited flexibility allowed at site II-site III junction, can be used to introduce foreign epitopes (Jallet *et al*., 1999). Since IFN-α/β plays an important role in directing the adaptive immune response, it may be a valuable molecular adjuvant in vaccine development. The use of IFN-β to promote induction of stronger $CD8^+$ T cell response might be beneficial in vaccine approaches. It has been shown that IFN-β works at the interface of innate and adaptive immune response by sustaining a pool of activated

antigen-specific $CD8^+$ lymphocytes (Faul *et al.*, 2008). Thus IFN-β directs cells of the adaptive immune system to appropriately respond to a pathogen. Moreover IFN β was also shown to reduce vector associated pathogenicity (Faul *et al.*, 2008). This could have a benefit as a vaccine candidate for use in immune-compromised populations. A second alternative would be expression of dual glycoproteins of MOKV and RABV. Introduction of a duplicate (second) glycoprotein gene has been shown to enhance immunogenicity through increased glycoprotein expression that presumably improved vaccine efficacy. The increased glycoprotein expression was associated with an up-regulation of IFN α/β and γ genes. However that study used a recombinant virus vector SPBN derived from SAD B19 cDNA clone and it would be interesting to establish if a vaccinia virus Copenhagen strain based vaccine vector would offer similar improvements.

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Appendix A:

Nucleotide sequence of PVPV recombinant using TKr primer

TTATCGCTGCATACGCTCACAGAATTCCCCGGGGAGCTCACTAGTGGATCCCTGCAGCTCGAGAGGCCTAA
TTAATTAAGTCGACTTTTTATCATATGCCCGGTAGTTGCGATATACATAAACTGATCACTAATTCCAAACC
CACCCGCTTTTTATAGTAAGTTTTTCACCCATAAATAATAAATACAATAATTAATTTCTCGTAAAAGTAG
AAAATATATTCTAATTTATTGCACGGTAAGGAAGTAGAATCATAAAGAACAGTGACGGATCGATCCCCCA
AGCTTGGACACAAGACAGGCTTGCGAGATATGTTTGAGAATACCACTTTATCCCGCGTCAGGGAGAGGCA
GTGCGTAAAAAGACGCGGACTCATGTGAAATACTGGTTTTTAGTGCGCCAGATCTCTATAATCTCGCGCAA
CCTATTTTCCCCTCGAACACTTTTTAAGCCGTAGATAAACAGGCTGGGACACTTCACATGAGCGAAAAATA
CATCGTCACCTGGGACATGTTGCAGATCCATGCACGTAAACTCGCAAGCCGACTGATGCCTTCTGAACAAT
GGAAAGGCATTATTGCCGTAAGCCGTGGCGGTCTGGTACCGGGTGCGTTACTGGCGCGTGAACTGGGTATT
CGTCATGTCGATACCGTTTGTATTTCCAGCTACGATCACGACAACCAGCGCGAGCTTAAAGTGCTGAAACG
CGCAGAAGGCGATGGCGAAGGCTTCATCGTTATTGATGACCTGGTGGATACCCGGTGGTACTGCGGTTGCG
ATTCGTGAAATGTATCCCAAAAGCGCACTTTGTCACCATCTTCGCAAAACCGGCTGGGTCGTCCGCTGGTT
GATGACTATGTTGTGATATCCCGCAGATACCTGGATGGAACAGCCGTGGGATATGGGCGTCGTATTCGTCC
CGCCAATCTCCAGTCGCTAGTCTTTTCCATCGGCTTGCACTGACTGGGCGTGTTCTTAACTTCAGGCGAGT
ACAATAGCTTCAGTAGATCCTGATGCTGCATCCATGACCAGTGACCTGAGTCGACTTGATCGAACTCGATA
AACATCC

ATG start codon: TKr: P 7.5 l/e promoter Eco gpt:

Nucleotide sequence of PVPV recombinant using TKl primer

GCTTCGGTAAGTCAGAATTAATTAGACGAGTTAGACGTTATCAAATAGCTCAATATAAATGCGTGACTAT
AAAATATTCTAACGATAATAGATACGGAACGGGACTATGGACGCATGATAAGAATAATTTTGAAGCATTG
GAAGCAACTAAACTATGTGATGTCTTGGAATCAATTACAGATTTCTCCGTGATAGGTATCGACATCTATA
TACTATATAGTAATACCAATACTCAAGACTACGAAACTGATACAATCTCTTATCATGTGGGTAATGTTCT
CGATGTCGATAGCCATATGCCCGGTAGTTGCGATATACATAAACTGATCACTAATTCCAAACCCACCCACT
TTTTATAGTAAGTTTTTCACCCATAAATAATAAATACAATAATTAATTTCTCGTAAAAGTAGAAAATATA
TTCTAATTTATTGCACGGTAAGGAAGTAGAATCATAAAGAACAGTCAGATCTGGATCTGCAGGTCGACGG
ATCCCAAGCTTCTTCTAGCCTCGAGAATTCGAGCTCGGTACCCTTCTAGAGCCACCATGGTTCCTCAGGCT
CTCCTGTTTGTACCCCTTCTGGTTTTTCCATTGTGTTTTGGGAAATTCCCTATTTACACGATACCAGACAA
GCTTGGTCCCTGGAGCCCGATTGACATACATCACCTCAGCTGCCCAAACAATTTGGTAGTGGAGGACGAAG
GATGCACCAACCTGTCAGGGTTCTCCTACATGGAACTTAAAGTTGGATACATCTCAGCCATAAAAATGAAC
GGGTTCACTTGCACAGGCGTTGTGACGGAGGCTGAAACCTACACTAACTTCGTTGGTTATGTCACAACCAC
GTTCAAAAGAAAGCATTTCCGCCCAACACCAGATGCATGTAGAGCCGCGTACAACTGGAAGATGGCCGGT
GACCCCAGATATGAAGAGTCTCTACACAATCCGTACCCTGACTACCACTGGCTTCGAACTGTAAAAACCAC
CAAAGGAGTCTCTCGTTATCATATCTCCAAGTGTGGCAGATTTGGACCCATATGACAGATCCCTTCACTCG
AGGGTCTTTCCTTGGCGGGAGTGCTCAGGAGTAGCGGGTGTCTTCTTACGTACTGCTCCACTACCACGATT
ACCCCATTGATGCCCGAGAATCCGAGACTAGGATGTCCTTGTGTGAATTTTTACCA

Rabies virus PV strain glycoprotein

Nucleotide sequence of MoKPV recombinant using TKr primer

TCCATCGATGCATACGCTCACAGAATTCCCCGGGGAGCTCACTAGTGGATCCCTGCAGCTCGAGAGGCCTA
ATTAATTAAGTCGACTTTTTATCATATGCCCGGTAGTTGCGATATACATAAACTGATCACTAATTCCAAA
CCCACCCGCTTTTTATAGTAAGTTTTTCACCCATAAATAATAAATACAATAATTAATTTCTCGTAAAAGT
AGAAAATATATTCTAATTTATTGCACGGTAAGGAAGTAGAATCATAAAGAACAGTGACGGATCGATCCCC
CAAGCTTGGACACAAGACAGGCTTGCGAGATATGTTTGAGAATACCACTTTATCCCGCGTCAGGGAGAGG
CAGTGCGTAAAAAGACGCGGACTCATGTGAAATACTGGTTTTTAGTGCGCCAGATCTCTATAATCTCGCGC
AACCTATTTTCCCCTCGAACACTTTTTAAGCCGTAGATAAACAGGCTGGGACACTTCACATGAGCGAAAAA
TACATCGTCACCTGGGACATGTTGCAGATCCATGCACGTAAACTCGCAAGCCGACTGATGCCTTCTGAACA
ATGGAAAGGCATTATTGCCGTAAGCCGTGGCGGTCTGGTACCGGGTGCGTTACTGGCGCGTGAACTGGGTA
TTCGTCATGTCGATACCGTTTGTATTTCCAGCTACGATCACGACAACCAGCGCGAGCTTAAAGTGCTGAAA
CGCGCAGAAGGCGATGGCGAAGGCTTCATCGTTATTGATGACCTGGTGGATACCGGTGGTACTGCGGTTGC
GATTCGTGAAATGTATCCAAAAGCGCACTTTGTCACCATCTTCGCAAAACCGGCTGGTCGTCCGCTGGTTG
ATGACTATGTTGTTGATATCCCGCAAGATACCTGGATTGAACAGCCGTGGGATATGGGCGTCGTATTCGTC
CCGCCAATCTCCGGTCGCTAATCTTTTCAACGCCTGGCACTGCCGGGCGTTGTTCTTTTTAACTTCAGCGG
GTTACATAGTTTCCAGTAAGTATTCTGAGCTGCATCCATGACACAGGCAACTGAGCGAAACCCTGTCAAAC
CCCGCTTAACATCTGAAGCTCGACGCTAGTCCGCCGCTTAATCACGGCCATACCGACTGTTGCGTCGACCT
TGATGTCACCATTCCTTAATGGATTC

ATG start codon: TKr: P7.5: gpt

Nucleotide sequence of MoKPV recombinant using TKl primer

CTTGGGTAAGTACAGAATTAATTAGACGAGTTAGACGTTATCAAATAGCTCAATATAAATGCGTGACTAT
AAAATATTCTAACGATAATAGATACGGAACGGGACTATGGACGCATGATAAGAATAATTTTGAAGCATTG
GAAGCAACTAAACTATGTGATGTCTTGGAATCAATTACAGATTTCTCCGTGATAGGTATCGACATCTATA
TACTATATAGTAATACCAATACTCAAGACTACGAAACTGATACAATCTCTTATCATGTGGGTAATGTTCT
CGATGTCGATAGCCATATGCCCGGTAGTTGCGATATACATAAACTGATCACTAATTCCAAACCCACCCACT
TTTTATAGTAAGTTTTTCACCCATAAATAATAAATACAATAATTAATTTCTCGTAAAAGTAGAAAATATA
TTCTAATTTATTGCACGGTAAGGAAGTAGAATCATAAAGAACAGTCAGATCTGGATCTGCAGGTCGAGAA
TTCACGCGTGGTACCTCTAGAATGAATATACCTTGCTTTGTTGTGATTCTCAGCTTAGCCACTACACATTC
TCTGGGAGAATTCCCCTTGTACACAATTCCTGAGAAGATAGAGAAATGGACTCCCATAGACATGATCCATC
TGAGTTGCCCCAACAACCTATTATCTGAGGAAGAAGGTTGCAATGCAGAGTCATCCTTTACTTACTTTGAG
CTCAAGAGTGGTTACCTAGCTCATCAGAAGGTTCCAGGGTTTACCTGTACCGGGGTCGTGAACGAGGCAGA
GACATATACAAACTTCGTCGGGTACGTCACCACAACCTTCAAAAGGAAGCACTTTAGGCCTACAGTAGCCG
CCTGTCGTGATGCCTACAACTGGAAAGTGTCAGGAGACCCCAGGTACGAAGAGTCACTCCACACTCCTTAT
CCTGACAGCAGTTGGTTGAGGACTGTGACTACAACCAAAGAATCACTTCTCATAATATCGCCCAGCATCGT
GGAAATGGATATTTACGGCAGGACTCTCCATTCCCCCATGTTTCCTTCAGGAGTATGTTCCAAGCTATATC
CCTCTGTCCCATCTGTGAGACTTATTCATGATTACCACATATGGCCTGGCCTGAAGAATCTAGCTGGAGTT
GGCCTGTGAATTCCTTTACTCAGCACGAGAGGCCATGACGGTCACGCATCTGT

ATG start codon: TKl: P 7.5 l/e promoter: Mokola glycoprotein

Appendix B:

Data used to determine neutralizing antibody titers:

VNAb titers to RABV after only primary immunization with PVPV recombinant vaccine. The titers are noted for day 10 (10 days after primary vaccination).

serum Tested	Titers [a]			mean	STDEV
	1st Replicate	2nd Replicate	3Rd Replicate		
C5A	1030	1060	1081	1057	25.63201
C5B	97	103	113	104.3333	8.082904
C5C	102.75	132	120	118.25	14.70332
C5D	195.7	210	221	208.9	12.68582
C5E	1250	1200	1230	1226.667	25.16611

[a] Titers reported as 10^ log (50 % End-point dilution) as determined with RFFIT

VNAb titers to RABV after immunization and boosting with PVPV recombinant vaccine. The titers are noted for day 28 (14 days after booster vaccination).

Serum Tested	Titers [a]			mean	STDEV
	1st Replicate	2nd Replicate	3Rd Replicate		
C6A	550	500	530	526.6667	25.16611
C6B	835	880	850	855	22.91288
C6C	1770.1	1660	1720	1716.7	55.12413
C6D	1770.1	1850	1800	1806.7	40.36917
C6E	1320.5	1400	1363	1361.167	39.7817

[a] Titers reported as 10^ log (50 % End-point dilution) as determined with RFFIT

VNAb titers to RABV after immunization and boosting with MokPV recombinant vaccine. The titers are noted for day 28 (14 days after booster vaccination).

serum Tested	Titers [a]			mean	STDEV
	1st Replicate	2nd Replicate	3Rd Replicate		
C3A	65	59	61	61.66667	3.05505
C3B	79	76	77.7	77.56667	1.504438
C3C	82	85	80.2	82.4	2.424871
C3D	94	94	94	94	0
C3E	97	101	100	99.33333	2.081666

[a] Titers reported as 10^ log (50 % End-point dilution) as determined with RFFIT

VNAb titers to MOKV after only primary immunization with MokG recombinant vaccine. The titers are noted for day 10 (10 days after primary vaccination).

serum Tested	Titers [a]			mean	STDEV
	1st Replicate	2nd Replicate	3Rd Replicate		
C8A	350.23	331	328	336.41	12.0621
C8B	225.4	198	210	211.1333	13.73511
C8C	196	187	193	192	4.582576
C8D	230.6	265	280	258.5333	25.32693
C8E	288	295	301	294.6667	6.506407

[a] Titers reported as 10^ log (50 % End-point dilution) as determined with RFFIT

VNAb titers to MOKV upon immunization and boosting with MokG recombinant vaccine. The titers are noted for day 28 (14 days after booster vaccination).

serum Tested	Titers [a]			mean	STDEV
	1st Replicate	2nd Replicate	3Rd Replicate		
C9A	1380.3	1250	1500	1376.767	125.0374
C9B	1548.7	1750	1300	1532.9	225.4157
C9C	1548.7	1400	1450	1466.233	75.66745
C9D	2001	2100	2600	2233.667	321.0924

C9E	1682.88	1340	2100	1707.627	269.1276

[a] Titers reported as 10^ log (50 % End-point dilution) as determined with RFFIT

VNAb titers to MOKV upon immunization and boosting with MokPV recombinant vaccine. The titers are noted for day 28 (14 days after booster vaccination).

serum Tested	Titers [a]			mean	STDEV
	1st Replicate	2nd Replicate	3Rd Replicate		
C3A	88	85	79	84	4.582576
C3B	103	94	88	95	7.549834
C3C	97	101	125	107.6667	15.14376
C3D	67	76	80	74.33333	6.658328
C3E	210	229	233	224	12.28821

[a] Titers reported as 10^ log (50 % End-point dilution) as determined with RFFIT

VNAb titers to LBV upon immunization and boosting with MokG recombinant vaccine. The titers are noted for day 28 (14 days after booster vaccination).

serum Tested	Titers [a]			Mean	STDEV
	1st Replicate	2nd Replicate	3Rd Replicate		
C9A	68	66.6	71.6	68.73333	2.579406
C9B	55	55	55.3	55.1	0.173205
C9C	59	63	65	62.33333	3.05505
C9D	62.8	62	58	60.93333	2.57164
C9E	76.8	74.2	79	76.66667	2.402776

[a] Titers reported as 10^ log (50 % End-point dilution) as determined with RFFIT

VNAb titers to LBV upon immunization and boosting with MokPV recombinant vaccine. The titers are noted for day 28 (14 days after booster vaccination).

serum Tested	Titers [a]			Mean	STDEV
	1st Replicate	2nd Replicate	3Rd Replicate		
C3A	55.53	50	61	55.51	5.500027
C3B	31.45	44	50	41.81667	9.465772
C3C	50	53	55	52.66667	2.516611
C3D	66.6	65	60	63.86667	3.442867
C3E	49	44	46	46.33333	2.516611

[a] Titers reported as 10^ log (50 % End-point dilution) as determined with RFFIT

Appendix C: Statistical representation of VNAb data

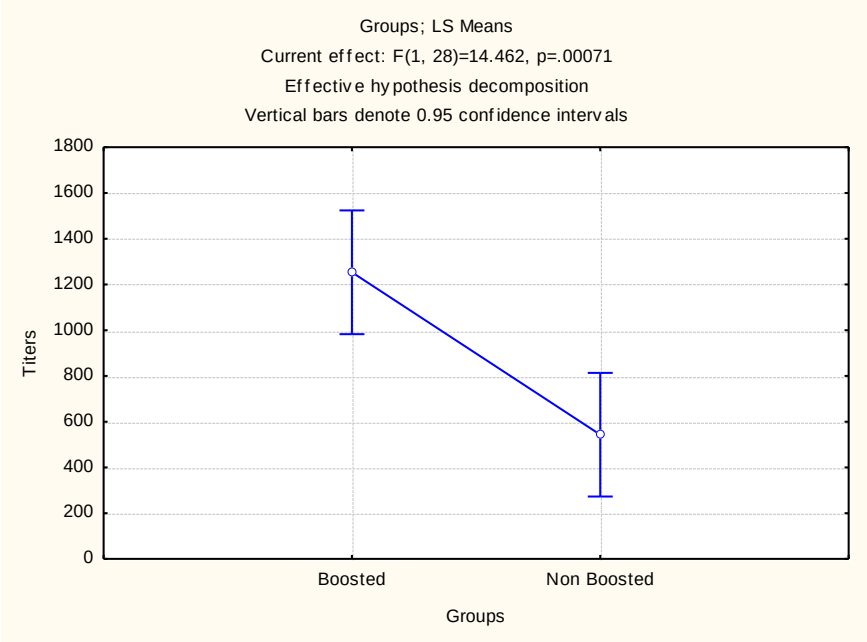


Figure C1: Neutralization response to CVS challenge with PVPV boosted vs non-boosted

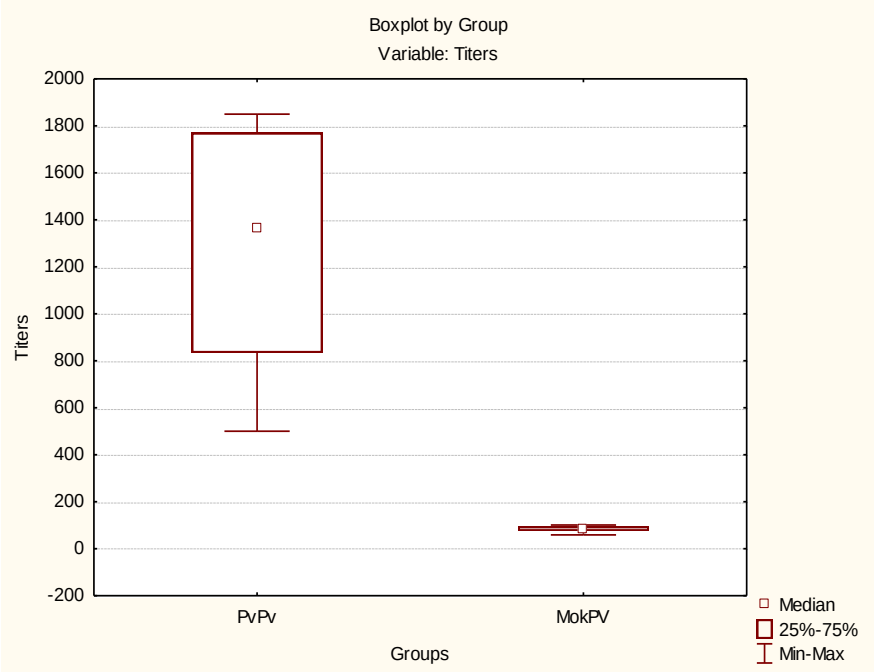


Figure C2: Neutralization response to CVS challenge with PVPV (boosted)_vs MokPV (boosted)

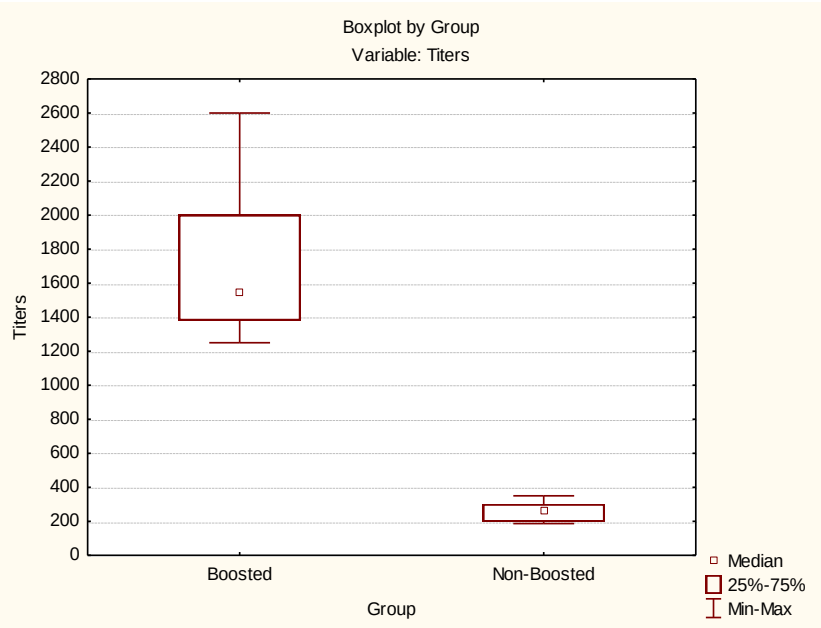


Figure C3: Neutralization response to MOKV with MokG boosted vs non-boosted

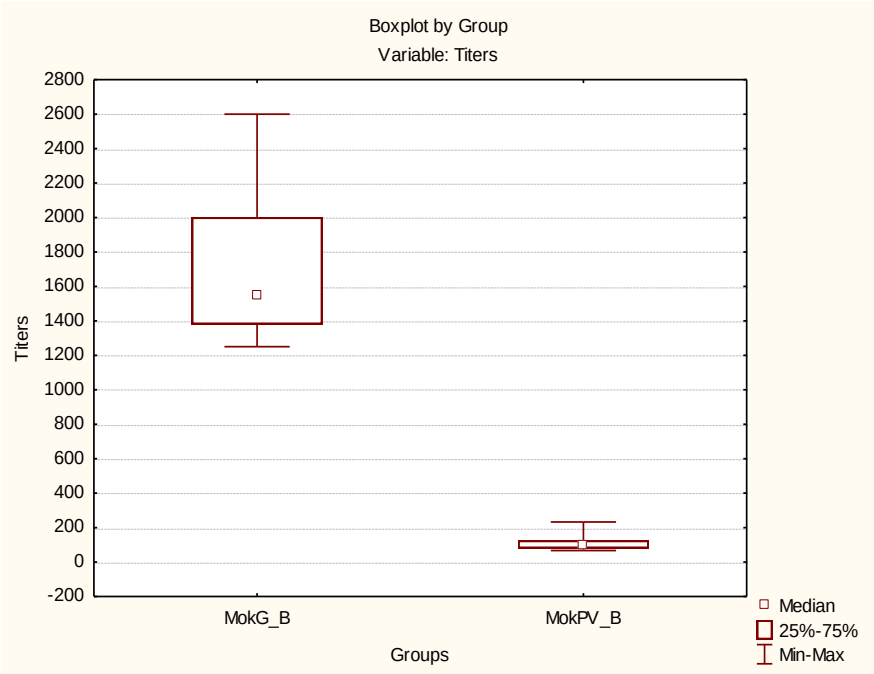


Figure C4: Neutralization response to MOKV with MokG boosted vs MokPV boosted

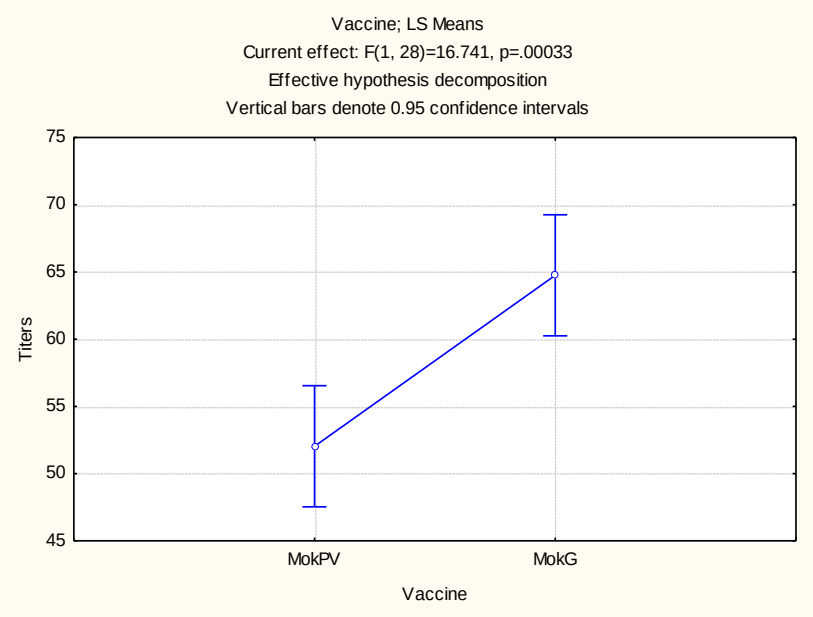


Figure C5: Neutralization response to Lagos bat virus with (MokPV boosted) vs (MokG boosted

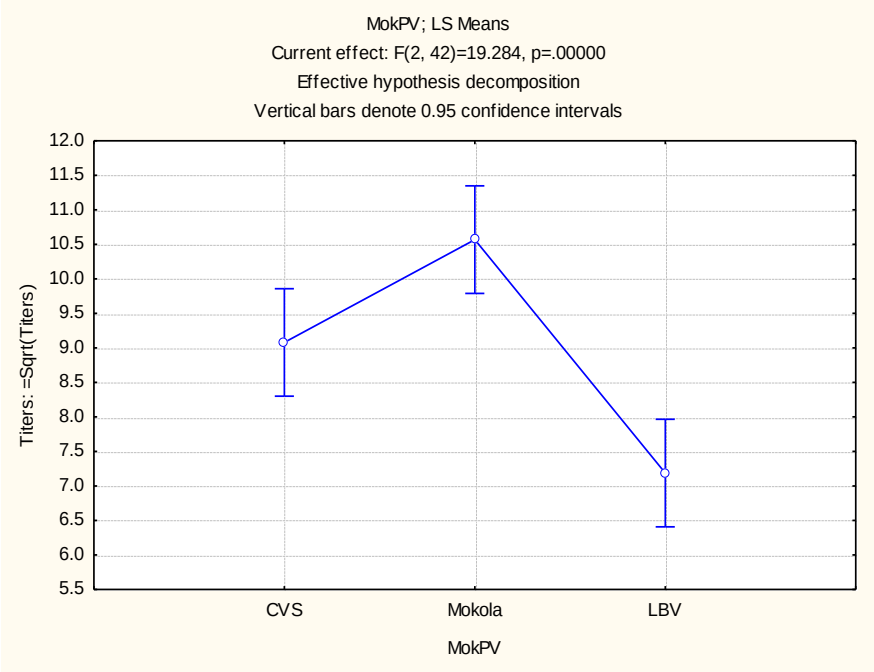
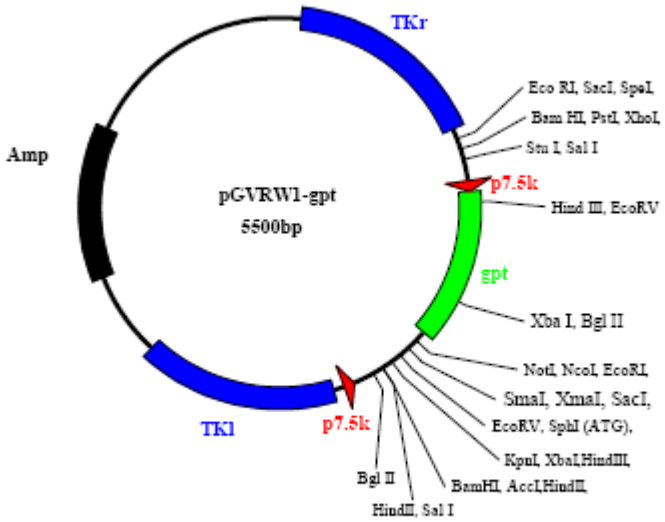


Figure C6: MokPV neutralization response to CSV, MOKV and LBV challenges

Appendix D: map of plasmid used in construction of recombinants



b: diagrammatic map of vaccinia virus transfer vector pGVWRI-gpt (adapted from Weyer, 2006)